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PAPEL DEL RECEPTOR HUÉRFANO GPR55 EN LA FISIOPATOLOGÍA DEL CÁNCER : IMPLICACIÓN EN LA PROLIFERACIÓN E INVASIÓN DE CÉLULAS TUMORALES

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
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Bajo la dirección de los doctores

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ABREVIATIONS

abn-CBD: abnormal cannabidiol

AC: adenylate cylcase

AEA: anandamide

2-AG: 2-arachidonoylglycerol

ANGPTL4: angiopoetin-like 4

AP1: activator protein-1

ATF-2: activating transcription factor 2

ATX: autotaxin

Bad: Bcl-2-associated death promoter

BAX: Bcl-2–associated X protein

Bcl-2: B-cell lymphoma 2

Bmx: bone marrow kinase X-linked

CAFs: cancer activated fibroblasts

CaMK: /Ca2+/calmodulin-dependent protein kinase

cAMP: cyclic adenosine monophosphate

CB_1: cannabinoid receptor type 1

CB_2: cannabinoid receptor type 2

CBD: cannabidiol

CCL: Chemokine (C-C motif) ligand

CCM: cholesterol consensus motif

CCR receptor: C-C chemokine receptor

Cdc42: cell division control protein 42

CNS: central nervous system

COX-2: cyclooxygenase-2

cPLA2: cytosolic phospholipase A2

CRAC: cholesterol recognition amino acid consensus

CXCL: Chemokine (C-X-C motif) ligand

CXCR receptor: C-X-C chemokine receptor

DAG: dyacilglycerol

ECL: extracellular loops

ECS: endocannabinoid system

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ERK: extracellular signal-regulated kinases

ET receptor: endothelin receptor

GAP: GTPase-activating proteins

GDP: guanosine diphosphate

GEF: guanine nucleotide exchange factor

GFP: green fluorescent protein

GIPs: G protein interacting proteins

GM-CSF: granulocyte macrophage-colony stimulating factor

GPCR: G protein coupled receptors

GRKs: G protein-coupled receptor kinase

GRO-α: growth-regulated oncogene-α

GRP: gastrin-releasing peptide

GTP: guanosine triphosphate

HEK293 cells: human embryonic kidney cells

HIF-1: hypoxia-inducible factor-1

ICL: intracellular loops

IL: interleukin

IFN-γ: interferon-γ

IP_3: inositol-1,4,5-triphosphate

JNK: c-Jun N-terminal kinases

KO: knock-out

LPA: lysophosphatidic acid

LPI: L-α-lysophosphatidylinositol

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

MMPs: matrix metalloproteinases

NFAT: nuclear factor of activated T-cells

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

NGF: nerve growth factor

PAR receptor: protease-activated receptor

PEA: palmitoylethanolamide

PGE2: prostaglandin E2

PI3K: phosphoinositide 3-kinase

PIP_2: phosphatidylinosito 4,5-biphosphate

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

p38 MAPK: p38 mitogenic-activated protein kinase

RGS: regulators of G-protein signaling

Rho: Ras homolog gene family

ROCK: Rho-associated protein kinase

α-SMA: α-smooth muscle actin

S1P: sphingosine 1-phosphate

SK: sphingosine kinsase

SPARC: secreted protein acidic and rich in cysteine

SRE: serum response factor

THC: tetrahydrocannabinol

TM: transmambrane

VEGF: vascular endothelial growth factor

VCAM1: vascular cell adhesion protein 1

WT: wild-type

RESUMEN

GPR55 es un receptor huérfano acoplado a proteinas G que, según publicaciones recientes, podría ser activado por lisofosfatidilinositol y determinados cannabinoides. Aunque se conoce poco todavía sobre su función fisiológica, existen evidencias que indican que podría desempeñar un papel importante en la regulación del metabolismo óseo o el control del dolor inflamatorio, por ejemplo. En esta Tesis hemos estudiado el papel de GPR55 en la fisiopatología del cáncer. Nuestros resultados muestran que GPR55 se expresa en tumores de distintos orígenes y que su expresión es significativamente más alta en tejidos transformados frente a tejidos sanos. Dicha expresión se correlaciona además con una mayor agresividad tumoral y un peor pronóstico de los pacientes. Los estudios que hemos realizado demuestran que GPR55 proporciona a las células cancerígenas una serie de ventajas adaptativas que podrían explicar la mayor agresividad de los tumores que expresan niveles elevados de GPR55. Primero, hemos demostrado que GPR55 induce la proliferación de células tumorales a través de la activación de la cascada de señalización ERK/MAPK, tanto en cultivos celulares como en modelos *in vivo* de cáncer. Esta activación promueve el crecimiento tumoral en modelos animales basados en xenografts y en el modelo de carcinogénesis química en piel de ratón. Segundo, hemos observado que la expresión de GPR55 favorece otra serie de características estrechamente relacionadas con la progresión tumoral en general y la generación de metástasis en particular. Así, GPR55 promueve el crecimiento independiente de anclaje, la migración e invasión de células tumorales a través de la activación de proteínas G_q y la generación *in vivo* de metástasis en pulmones.

Por último, hemos investigado si GPR55 está implicado en el efecto bifásico de los cannabinoides sobre la proliferación de células tumorales. Nuestros resultados muestran que GPR55 participa en la respuesta pro-proliferativa que induce el THC a bajas concentraciones, y sugieren que dicha acción podría estar mediada por la interacción funcional entre GPR55 y el receptor clásico de cannabinoides CB_1.

En conjunto, estos resultados sugieren que GPR55 podría ser un nuevo marcador con valor pronóstico y una nueva diana terapéutica en oncología.

ABSTRACT

GPR55 is an orphan G protein-coupled receptor that has been proposed to be engaged by lysophospholipids and cannabinoids. Though little is known about the physiological function of GPR55, emerging evidences point to important regulatory functions throughout the body. In this Thesis we studied the role of GPR55 in cancer physio-pathology. We found that GPR55 is expressed in human tumors from different origins, and that this expression is higher in the transformed tissues as compared with the corresponding non-transformed tissues and correlates with tumor aggressiveness. Our results show that GPR55 expression confers a series of adaptative advantages on cancer cells that make them more aggressive. First, we demonstrate that GPR55 drives cancer cell proliferation, through activation of the ERK/MAPK cascade, both in cell cultures and *in vivo*, which results in the promotion of tumor growth in xenograft-based and chemical-carcinogenesis animal models of cancer. Second, we show that GPR55 expression favors another set of capabilities that are intimately related to tumor progression in general and the generation of metastasis in particular. Thus, GPR55 promotes anchorage-independent growth, migration and invasion of cancer cells in culture through activation of G_q proteins and the generation of lung metastasis *in vivo*.

Finally, we investigated if GPR55 is involved in the biphasic effect of cannabinoids on cancer cell proliferation (low concentrations producing pro-proliferative responses while high concentrations inducing anti-proliferative effects). Our data show that GPR55 modulates the proliferation-inducing response produced by low concentrations of cannabinoids, and suggest that this action may be produced by functional interaction of GPR55 with the classical cannabinoid receptor CB_1.

Together, our data suggests that GPR55 could be a new biomarker with prognostic value and a therapeutic target in oncology.

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RESUMEN

Los receptores acoplados a proteínas G (GPCRs) constituyen la superfamilia más numerosa de receptores celulares. Controlan funciones fisiológicas cruciales, y su disfunción contribuye al desarrollo y aparición de muchas enfermedades humanas, incluyendo el cáncer. Por ello, junto con las enzimas, son las dianas terapéuticas más comunes.

Los GPCRs son proteínas integrales de membrana, constituidas por una dominio extracelular N-terminal, 7 α-hélices transmembranales (7TM) conectadas por 3 loops extracelulares (ECL1, ECL2 y ECL3) y 3 loops intracelulares (ICL1, ICL2, ICL3), acabando en un dominio intracelular C-terminal. Los GPCRs transforman un estímulo externo (fotones, neurotransmisores, quimioquinas, mediadores lipídicos, hormonas) en señales intracelulares que desencadenarán respuestas biológicas.

El cáncer es una de las enfermedades más comunes en el mundo, y una de las primeras causas de muerte en los países desarrollados. En las últimas décadas su incidencia ha ido aumentado, y estudios de la Organización Mundial de la Salud indican un continuo crecimiento en todo el mundo en los años venideros. El cáncer es un proceso evolutivo que conlleva una serie de cambios dinámicos en el genoma que producen la acumulación de mutaciones somáticas. Durante este proceso, las células normales adquieren diferentes capacidades que desencadenarán su transformación maligna. Estas capacidades se conocen como las “Bases del cáncer”. Diferentes estudios han demostrado que los GPCRs pueden modular muchos de estos procesos, si no todos. Así, los GPCRs favorecen la proliferación sin control de células tumorales, su resistencia a apoptosis y su capacidad invasiva y metastática así como la angiogénesis y el control del microambiente tumoral.

El amplio espectro de acción de estos GPCRs en la fisiopatología del cáncer les convierte en potenciales dianas terapéuticas. A pesar de ello, solo una pequeña proporción de GPCRs se están explotando clínicamente en la actualidad en oncología. Conocer el mecanismo de acción de los GPCRs en la iniciación y progresión tumoral es crucial por tanto para incrementar las herramientas terapéuticas basadas en estos receptores. Los GPCRs huérfanos no tienen un ligando endógeno asignado y sus funciones fisiológicas suelen ser desconocidas, lo que los convierte en una población muy atractiva para estos estudios. GPR55 es un receptor huérfano que fue identificado y clonado por primera vez en 1999. Distintos estudios sugieren que el L-α-lisofosfatidilinositol (LPI) es el ligando endógeno de GPR55, y que diferentes compuestos cannabinoides pueden unirse y activar dicho receptor. GPR55 tiene una expresión muy ubicua, y, aunque se conoce poco sobre su papel fisiológico y mecanismos de acción, se ha propuesto que regula diferentes respuestas biológicas en el sistema nervioso central, hueso, sistema inmune y vascular, entre otros.

Diferentes evidencias indirectas sugieren que GPR55 podría estar implicado en la fisiopatología del cáncer. Primero, tanto el plasma como el líquido ascítico de pacientes con cáncer de ovario presentan altos niveles de LPI, el ligando endógenos propuesto para GPR55, comparado con el plasma y líquido

ascítico de mujeres sin una patología oncológica. Segundo, GPR55 se une a las proteínas heterotriméricas $G_{12/13}$ y G_q, que tienen propiedades oncogénicas, y señaliza a través de Rho GTPasas, proteínas que controlan la organización del citoesqueleto, la polaridad celular y la migración, procesos intimamente relacionados con la progresión tumoral. Por último, determinados cannabinoides (compuestos con propiedades anti-tumorales) pueden activar GPR55.

Considerando todas estas evidencias, la **HIPÓTESIS** de trabajo de esta Tesis es que GPR55 participa en el control de la fisiopatología del cáncer. Los **OBJETIVOS** específicos del trabajo son:

1. Estudiar la expresión de GPR55 en distintos tumores humanos.

2. Investigar el papel de GPR55 en la generación y progresión tumoral.

3. Analizar si GPR55 está implicado en el efecto de los cannabinoides sobre la proliferación de células tumorales.

Los **RESULTADOS** obtenidos en esta Tesis se han dividido en cuatro capítulos:

En el **PRIMER CAPÍTULO** hemos intentado determinar si GPR55 juega un papel importante en la fisiopatología del cáncer. Para ello, primero hemos analizado la expresión de GPR55 en una colección de líneas celulares humanas y muestras tumorales de pacientes con cáncer de mama, glioblastoma y adenocarcinomas de páncreas. Además, hemos modulado la expresión de GPR55 en diferentes líneas tumorales humanas (mediante transfección con un vector que contiene la construcción HA-GPR55 o mediante silenciamiento genético del receptor a través de siRNAs específicos) para determinar la implicación de GPR55 en la proliferación de células tumorales *in vitro* e *in vivo*. También hemos caracterizado los mecanismos moleculares responsables de estos efectos. Hemos observado que la expresión del receptor está aumentada significativamente en tejido tumoral frente a tejido sano, y en tumores altamente agresivos frente a poco agresivos. Además, GPR55 promueve la proliferación de células tumorales tanto en cultivos celulares como en modelos animales basados en *xenografts* a través de la activación de la cascada de señalización de ERK/MAPK.

Estos resultados revelan un papel importante de GPR55 en cáncer, y sugieren que podría ser un posible nuevo marcador biológico y una nueva diana terapéutica en oncología.

En el **SEGUNDO CAPÍTULO**, hemos estudiado en más detalle el papel de GPR55 en la generación y progresión tumoral. Para alcanzar este objetivo, hemos utilizado el modelo de carcinogénesis química en piel de ratón en animales que no expresan el receptor GPR55 y sus correspondientes hermanos salvajes. Hemos estudiado el efecto de la ablación genética de GPR55 en la transformación maligna, y hemos intentado revelar las bases moleculares de dichos efectos. Finalmente, hemos analizado la expresión de GPR55 en diferentes carcinomas de célula escamosa humanos, y hemos intentado deter-

minar si la expresión de este receptor se correlaciona con marcadores con relevancia clínica. Nuestros resultados demuestran que GPR55 induce el desarrollo de tumores de piel en ratón. Así, los ratones deficientes en GPR55 son más resistentes a la aparición de papilomas inducidos por DMBA/TPA y a la transformación de papilomas en carcinomas que sus hermanos salvajes. Según nuestros resultados, GPR55 produce estos efectos pro-tumorales proporcionando una ventaja proliferativa a las células tumorales, mediante la sobre-activación de la cascada de señalización ERK/c-Fos/ciclina D1. Nuestros resultados también muestran que GPR55 proporciona otras ventajas oncogénicas en las células tumorales de piel. Por ejemplo, GPR55 promueve el crecimiento independiente de anclaje, la invasión y la tumorigenicidad *in vivo*, lo que sugiere que este receptor, además de promover el desarrollo tumoral, incrementa la agresividad de los tumores. Por último hemos observado que GPR55 se encuentra sobre-expresado en tumores de piel humanos y en otros carcinomas de célula escamosa con respecto al tejido sano del mismo paciente.

En conjunto, estos resultados revelan un papel importante de GPR55 en el desarrollo de tumores de piel, y refuerzan la idea de que este receptor podría ser un nuevo marcador y diana terapéutica también para carcinomas de célula escamosa.

En el **TERCER CAPÍTULO** de esta Tesis, hemos investigado si GPR55 participa en el proceso metastático. Así, hemos analizado el efecto de GPR55 en la migración e invasión *in vitro* de células de cáncer de mama así como el crecimiento de tumores y la colonización en pulmón *in vivo*. Para estos estudios hemos utilizado células tumorales humanas en las que se ha silenciado de manera estable GPR55 (o las correspondientes células control) y hemos estudiado los mecanismos responsables de las características pro-metastáticas inducidas por GPR55. Nuestros resultados demuestran que GPR55 induce la migración e invasión de las células de cáncer de mama altamente metastáticas MDA-MB-231. Estas células expresan altos niveles de GPR55, y el silenciamiento del receptor reduce su invasividad hacia suero y LPI (el ligando endógeno propuesto para GPR55). Más aún, cuando la expresión de GPR55 es recuperada, las células tumorales aumentan su invasividad hacia ambos quimioatrayentes. Como se ha descrito anteriormente, GPR55 se une a proteínas $G_{12/13}$ y G_q, proteínas heterotriméricas implicadas en la invasión y metástasis de células tumorales. Utilizando dominantes negativos de estas proteínas G, hemos observado que GPR55 promueve la invasión vía G_q y que la activación de MMPs y el aumento de expresión de genes inductores de metástasis están también implicados en el efecto pro-invasivos inducido por GPR55. Por último, nuestros resultados muestran que GPR55 promueve el crecimiento tumoral y la colonización pulmonar *in vivo*.

En conjunto, estos resultados apoyan que la implicación de GPR55 en el último y más letal paso en la progresión tumoral, y sugieren que su bloqueo farmacológico podría ser una nueva estrategia para el tratamiento del cáncer metastático.

Como se ha descrito anteriormente, publicaciones recientes demuestran que GPR55 puede ser activado por diferentes compuestos cannabinoides. Es bien conocido que los cannabinoides controlan la

proliferación celular. En células tumorales estos compuestos generalmente inducen un efecto bifásico: a bajas concentraciones estimulan la proliferación celular mientras que a altas concentraciones producen efecto anti-proliferativo. En este contexto, en el **CUARTO CAPÍTULO** de la Tesis hemos estudiado si GPR55 media el efecto del THC sobre la proliferación de células tumorales. Primero hemos analizado si la modulación de la expresión de GPR55 altera la respuesta al THC, y a continuación hemos intentado revelar cómo se controlan molecularmente estas respuestas. En concreto hemos estudiado si el THC induce liberación de calcio intracelular vía GPR55 y si el efecto del THC es producido por interacción de GPR55 con receptores de cannabinoides clásicos. Nuestros resultados demuestran que GPR55 media el efecto pro-proliferativo inducido por concentraciones bajas de THC. Así, el silenciamiento de GPR55 en células de glioblastoma y de adenocarcinoma de mama bloquea el pico proliferativo inducido por THC, mientras que la sobreexpresión del receptor incrementa la proliferación celular. Además, nuestros datos demuestran la existencia de una interacción funcional entre GPR55 y CB1, y sugieren que distintos estados de interacción GPR55 /CB1 podrían ser la causa del efecto bifásico del THC sobre la proliferación de células tumorales.

En resumen, las **CONCLUSIONES** de esta Tesis son:

1. El receptor huérfano GPR55 proporciona ventajas pro-oncogénicas a las células tumorales, induciendo su proliferación, migración e invasión tanto *in vitro* como *in vivo*. En consecuencia, niveles elevados de GPR55 en tumores humanos se correlacionan con una mayor agresividad tumoral y un peor pronóstico de los pacientes.

2. GPR55 participa en el efecto del THC sobre la proliferación de células tumorales, concretamente en el aumento de la proliferación inducido por bajas concentraciones del cannabinoide.

En conjunto, nuestros datos sugieren que GPR55 podría ser una nueva herramienta con valor pronóstico y una nueva diana terapéutica en cáncer.

SUMMARY

G protein-couple receptors (GPCRs) constitute the largest superfamily of cellular receptors. They control crucial physiological functions and, consequently, their dysfunction contributes to many human diseases including cancer. In fact, they are -together with enzymes- the most common target of therapeutic drugs.

GPCRs are integral membrane proteins, characterized by an extracellular N-terminal domain, 7 transmembrane α-helices (7-TM) connected by 3 extracellular loops (ECL1, ECL2 and ECL3) and 3 intracellular loops (ICL1, ICL2 and ICL3), ending in an intracellular C-terminal domain. They transform external stimuli (photons, neurotransmitters, chemokines, lipid mediators, hormones) into intracellular signals that will eventually trigger biological responses.

Cancer is one of the most common diseases worldwide and one of the firsts causes of death in developed countries. Its incidence has grown in the past decades, and studies from the World Health Organization point to a continuous increase over the world in the coming years. Cancer is an evolutionary process that involves dynamic changes in the genome that result in accumulation of somatic mutations. During this process, normal cells acquire different capabilities that lead to malignant transformation. These capabilities are known as the “hallmarks of cancer”. Different studies have shown that GPCRs can modulate most, if not all, these hallmarks. Thus, GPCRs participate in favoring uncontrolled cell proliferation, resistance to apoptosis, sustained angiogenesis, enhanceed tissue invasion and metastasis, and control tumor microenvironment.

The wide spectrum of action of GPCRs in cancer physiopathology makes them potential anti-tumoral targets. However only few GPCRs are currently being therapeutically exploited in oncology. Understanding the mechanism of action of GPCRs in cancer initiation and progression is therefore crucial to increase the therapeutic armamentarium based on these receptors. Orphan GPCRs, with no endogenous ligand assigned yet and whose physiological functions are in most cases unknown, constitute a very attractive population for such studies. GPR55 is an orphan GPCR that was first identified and cloned in 1999. Several reports suggest that L-α-lysophosphatidylinositol (LPI) is the potential endogenous ligand for GPR55, and that different cannabinoid compounds can bind to and activate this receptor. GPR55 is widely express throughout the body, and, although little is still known about the physiological relevance and mechanism of action of the receptor, it has been proposed to regulate different physiological responses in the central nervous system, bone, immune system and vasculature, among others.

Different indirect evidences suggest that GPR55 may be involved in cancer physiopathology. First, increased levels of LPI, the putative GPR55 endogenous ligand, have been found in plasma and ascites from patients with ovarian cancer compared with women without oncogenic pathologies. Second, GPR55 couples to $G_{12/13}$ and G_q, which are known to have oncogenic properties, and signals through Rho GTPases which control cytoskeleton organization, cell polarity and cell migration, all of them inti-

matelly related to tumor progression. Finally, certain cannabinoids (compounds with known antitumoral actions) can activate GPR55.

In this context, the working **HYPOTHESIS** of this Thesis is that GPR55 participates in the control of tumor physio-pathology. In this context, the specific **AIMS** of this project are:

1. To study the expression of GPR55 in human tumors.

2. To investigate the role of GPR55 in tumor generation and progression.

3. To analyse whether GPR55 is involved in the effect of cannabinoids on cancer cell proliferation.

The **RESULTS** obtained in this Thesis are divided in four chapters.

In the **FIRST CHAPTER** we tried to determine whether GPR55 plays any significant role in cancer physiopathology. First, we analyzed the expression of GPR55 in a collection of human tumor cell lines and human breast cancer, glioblastoma and pancreatic adenocarcinoma samples. In addition, we modulated GPR55 expression in cancer cell lines (by transfection with HA-GPR55 constructions or genetic knock-down with specific siRNAs) to determine the involvement of GPR55 in cancer cell proliferation both *in vitro* and *in vivo*. We also characterized the molecular mechanisms underlying GPR55 action on cancer cells. We described that GPR55 is expressed in a wide variety of human cancers. In particular, we found that the expression of the receptor was significantly increased in tumoral tissues as compared with healthy tissues, and in highly aggressive vs poorly aggressive tumors. Moreover, GPR55 promotes cancer cell proliferation both in cell cultures and in xenografted mice through the overactivation of the extracellular signal-regulated kinase (ERK)/MAPK cascade.

These findings reveal the importance of GPR55 in human cancer, and suggest that it could constitute a new biomarker and therapeutic target in oncology.

In the **SECOND CHAPTER**, we studied in more detail the role of GPR55 in tumor generation and progression. To achieve this goal, we used the well-established model of chemically-induced skin carcinogenesis in mice lacking GPR55 and their corresponding wild-type littermates. We studied the effects of deleting GPR55 on malignant transformation, and we tried to unravel the molecular bases underlying such effects. Finally, we analyzed the expression of GPR55 in different human squamous cell carcinomas and we tried to determine whether this expression correlates with markers with clinical relevance. We demonstrated that GPR55 drives mouse skin tumor development. Thus, GPR55-deficient mice were more resistant to DMBA/TPA-induced papillomas, as indicated by a reduced generation of papillomas per animal than their corresponding wild-type littermates, and a decreased percentage of papilloma-carcinoma conversion. GPR55 exerted this pro-tumor effect primarily by conferring a proliferative advantage on cancer cells, via overactivation of the ERK/c-Fos/cyclin D1 cascade. Our data also shows that

GPR55 confers other oncogenic advantages on skin cancer cells. Thus, GPR55 enhances cancer cell anchorage-independent growth, invasiveness and tumorigenicity *in vivo*, suggesting that it not only promotes tumor development but also enhances tumor aggressiveness. Finally, we observed that GPR55 is upregulated in human skin tumors and other human squamous cell carcinomas compared with the corresponding healthy tissues.

Altogether, these findings reveal the pivotal importance of GPR55 in skin tumor development, and suggest that this receptor may be used as a new biomarker and therapeutic target also in squamous cell carcinomas.

In the **THIRD CHAPTER** of this Thesis, we investigated whether GPR55 participates in the metastatic process. Thus, we analyzed the effect of GPR55 on breast cancer cell migration and invasion *in vitro* and in tumor growth and lung colonization *in vivo*. For these studies we used breast cancer cells with stably knocked-down GPR55 levels or transfected with the corresponding control. We studied the downstream mechanisms responsible for the effects of GPR55 on the pro-metastasis-related features. Our results show that GPR55 induces the migration and invasion of the highly metastatic breast cancer cell line MDA-MB-231. This cell line expresses high levels of GPR55, and receptor donwregulation reduces the invasiveness of these cancer cells toward FBS and LPI, the proposed endogenous ligand of GPR55. Moreover, when GPR55 expression was reconstituted, tumor cells increased their invasion towards both chemoatracttants. As described before, GPR55 couples to $G_{12/13}$ and G_q proteins, heterotrimeric G proteins that have been implicated in tumor invasion and metastasis. By using negative dominants of the G proteins, we demonstrated that GPR55 drives breast cancer cell invasion via G_q signaling, and our results suggest that activation of MMPs and the upregulation of metastasis-inducing genes are also involved in the effect of GPR55 on cancer cell invasion and migration. Finally, our data show that GPR55 promotes breast cancer tumor growth and lung colonization *in vivo*.

Together, these results support the involvement of GPR55 on the latest and most lethal stage of cancer progression, and suggest that its pharmacological blockade could be a new strategy to manage metastatic cancer.

Recent reports have shown that GPR55 can be activated by different cannabinoid compounds. In addition, it is well established that cannabinoids control cell proliferation. These compounds generally induce a biphasic effect: while low concentrations induce cancer cell proliferation, high concentrations exert their well-known anti-proliferative action. Thus, in the **FOURTH CHAPTER** we studied whether GPR55 mediates the effects of THC on cancer cell proliferation. First, we analyzed the consequence of modulating GPR55 expression on THC action. Then, we tried to unravel how THC produces such effects via GPR55. In particular, we studied whether THC promotes a release of intracellular calcium via GPR55 and whether THC action is produced by functional interaction of GPR55 with classical cannabinoid receptors. We show that GPR55 mediates cancer cell proliferation induced by low concentrations of THC. Thus, knocked-down of GPR55 in glioblastoma and breast adenocarcinoma cells blocked the

pro-proliferative effect induced by THC, while overexpression of the receptor enhanced proliferation. Moreover, our data show that there is a functional interaction between GPR55 and CB_1 cannabinoid receptors, and suggests that the interaction status of these receptors may underlie the biphasic effects of THC on cell proliferation.

In summary, the **CONCLUSIONS** of this Thesis are:

1- The orphan receptor GPR55 confers pro-oncogenic advantages on tumor cells *in vitro* and in mouse cancer models by promoting their proliferation, migration and invasion. Consequently, elevated expression of GPR55 in human tumors is associated to high aggressiveness and poor prognosis.

2- GPR55 participates in the proliferative effect of THC on cancer cells as produced by low concentrations of the cannabinoid.

Together, these conclusions point to GPR55 as a new biomarker, with possible prognostic value, and as a new therapeutic target in oncology.

INTRODUCTION

1. G protein-coupled receptors

In a biological system, the interaction between the external environment and the inside is crucial. In cells, this important role is mostly mediated by proteins present in their membranes. G protein-coupled receptors (GPCRs) constitute the largest superfamily of such proteins, with more than 800 members in the human genome. They transform external stimuli (photons, neurotransmitters, chemokines, lipid mediators, hormones) into intracellular signals that will eventually trigger biological responses. These responses modulate a wide range of physiological functions such as vision, neurotransmission, cell proliferation, blood pressure, olfaction and taste (Pierce et al., 2002; Rosenbaum et al., 2009).

GPCR structure

GPCRs have been known for more than 40 years (Hill, 2009). The first 3D structure corresponding to a GPCR-related protein, the proton pump bacteriorhodopsin, was solved in 1975 from *Halobacterium halobium* using electron microscopy (Henderson and Unwin, 1975). However, it wasn't until 2000 when the first X-ray high-resolution structure of a GPCR was determined, in this case, of the inactive visual receptor rhodopsin (Palczewski et al., 2000). Another 7 years where needed to solve the structure of a GPCR bound to a ligand, the β_2-adrenergic receptor bound to its antagonist calazolol (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007).

GPCRs are integral membrane proteins, characterized by an extracellular N-terminal domain,

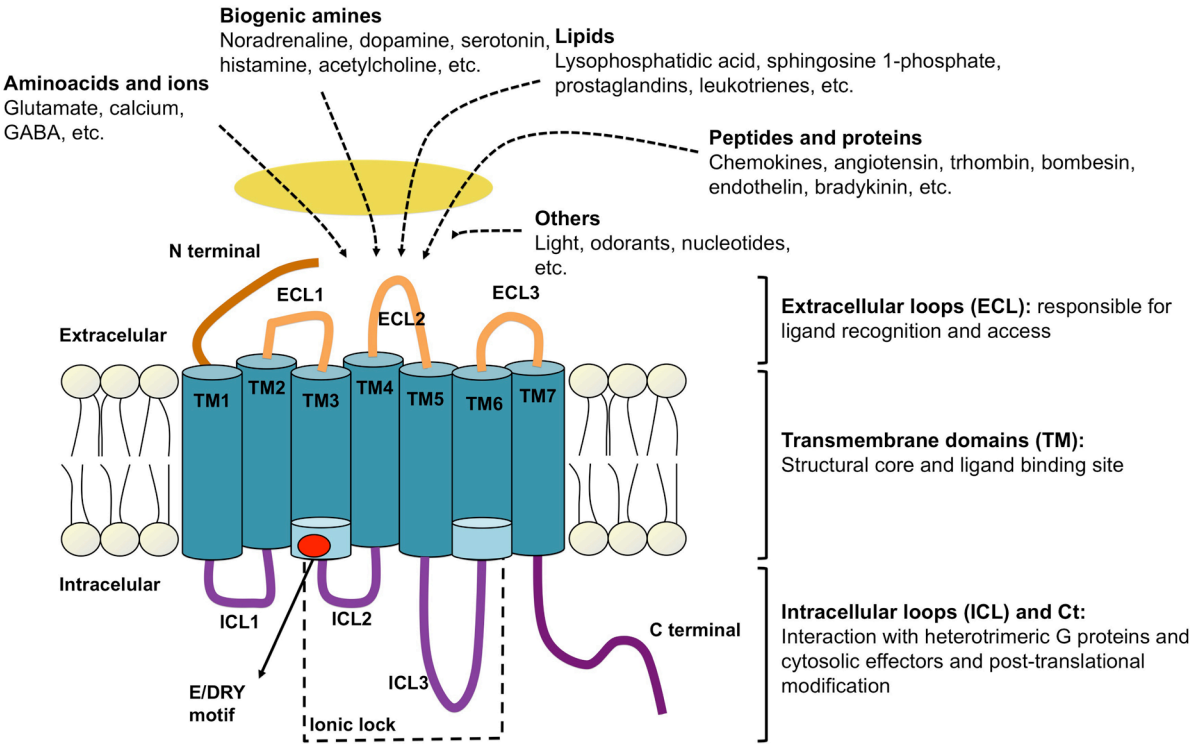

Figure 1. GPCR structure. *GPRCs are integral proteins formed by an extracellular N-terminal domain, 7 transmembrane α-helices (7TM) concerted by 3 extracellular loops (ECLS) and 3 intracellular loops (ICL), ending in an intracellular C-terminal. GPCRs can be activated by a wide variety of stimuli. These ligands are recognized mainly by the ECLs, interact with the TM domains (in the so called binding pocket), activate the receptor producing a conformational change that allows it to interact with downstream effectors (mainly, but not only, heterotrimeric G proteins).*

7 transmembrane α-helices (7-TM) connected by 3 extracellular loops (ECL1, ECL2 and ECL3) and 3 intracellular loops (ICL1, ICL2 and ICL3), ending in an intracellular C-terminal domain (figure 1) (Audet and Bouvier, 2012; Venkatakrishnan et al., 2013). They can be grouped into five major classes on the basis of their sequence and structural similarity: rhodopsin-like (class A), secretin-like (class B), metabotropic glutamate-like (class C), adhesion and Frizzled/Taste2 (figure 2) (Audet and Bouvier, 2012; Rosenbaum et al., 2009). Most of the GPCRs belong to the classes A, B and C, being the rhodopsin-like family the largest and most diverse, with conserved sequence motifs that imply shared structural features and activation mechanisms.

The principal structure studies have been made in the class A receptors. The members of this class differ in their extracellular loops and ligand-binding regions while the folding of the TM domains is highly conserved. The extracellular loops, mainly ECL2, are responsible for ligand recognition and access into the binding pocket in the TM region (Venkatakrishnan et al., 2013). The TM region is the structural core of the receptor where ligands bind ("ligand-binding pocket") and is in charge of transducing the information to the intracellular region. TM3 is essential for GPCR structure and function, and presents consensus contacts with TM2, TM4, TM5 and TM6. TM3 has a conserved motif (E/DRY motif) that regulates GPCR conformational state. The triplet of amino acids that constitute this motif (Glu/Asp-Arg-Tyr) form a network of polar interactions with amino acids in TM6 creating an "ionic lock" that stabilizes the inactive-state of the receptor. When ligands access the binding pocket, they interact with residues from TM3, TM6 and TM7, occupying similar spaces. The variation on the amino acids

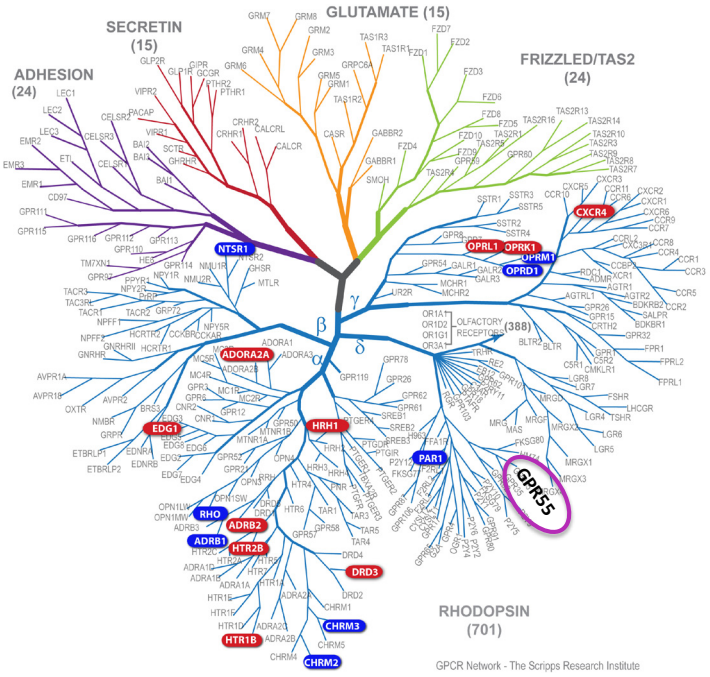

Figure 2. GPCR network. *Classification of GPCRs in the five major classes: Rhodopsin, adhesion, secretin, glutamate and Frizzled/Tas2. The GPCR studied in this Thesis, GPR55, is highlighted.*

in these regions provides ligand specificity (Audet and Bouvier, 2012; Millar and Newton, 2009). Ligand binding produces a conformational change in the TM core that leads to the disruption of the interactions in the "ionic lock" and the subsequent rearrangement of TM6 and the associated intracellular loop. This movement allows the interaction of ICL2 with the E/DRY motif in TM3, favoring receptor stability and generating a pocket for interaction with heterotrimeric G proteins and other intracellular effectors (Audet and Bouvier, 2012; Millar and Newton, 2009; Rosenbaum et al., 2009; Venkatakrishnan et al., 2013). Peptide motifs present in ICL3 and the C-terminal domain determine the specific partner that will interact with each GPCR. The C-terminal region also contains sequences susceptible of post-translational modification (phosphorylation associated to desensitation, palmitoylation for lipid raft recognition, etc.) (Venkatakrishnan et al., 2013) (figure 1).

It has been classically accepted that GPCR activation occurs according to the two-state model.

In this model, the receptor presents two conformations, the inactive (R) state, that has low affinity for agonists, versus the active (R*) state that binds agonists with high affinity and couples to G proteins activating downstream signaling pathways. The receptor exists in an equilibrium between these two states, and agonist binding displaces the equilibrium to the R* state (Gether and Kobilka, 1998; Park et al., 2007). Emerging evidences point to a highly dynamic and more complex scenario where the receptor could be in different conformational states (not only R and R*), that would lead to the modulation of different intracellular signaling pathways (Park, 2012; Venkatakrishnan et al., 2013).

It was generally admitted that GPCRs function as monomers, but accumulating evidence support that GPCRs, like other cell surface receptors, can form dimers or oligomers. Indeed, biochemical, biophysical and functional analyses demonstrate that GPCRs are expressed in the cell surface as oligomers (Audigier et al., 2013; Pierce et al., 2002; Vischer et al., 2011). These receptor interactions have profound consequences on cell signaling. For example, heterodimerization is required in some cases to produce the activation of particular effects [dimerization of $GABA_{B1}$ and $GABA_{B2}$ receptors for receptor signaling (Pin et al., 2004); dimerization of taste receptors $T1R_3$ with $T1R_1$ or $T1R_2$ for umami and sweet sensations, respectively (Zhao et al., 2003); dimerization of β_2-adrenergic with α_{1D}-adrenergic receptors for receptor cell surface targeting (Uberti et al., 2005), etc.]. In other cases, receptor heterodimerization modulates the functional features of the individual monomers. For example, activation of $mGlu_2$ in heteromers with $5\text{-}HT_{2A}$ increases the affinity of this latter receptor for its ligand (Gonzalez-Maeso et al., 2008). Another example are D_1-D_2 dopamine receptor heteromers, which couple to G_q while D_1 and D_2 in their monomeric forms signal through G_s and G_i proteins, respectively (Lee et al., 2004; Rashid et al., 2007).

GPCR signaling pathways

The canonic GPCR-activated signaling pathway relies on the activation of heterotrimeric G proteins, although they can also signal via non-G proteins.

Heterotrimeric G protein pathway

Heterotrimeric G proteins, also called guanine nucleotide-binding proteins, are GTPases formed by three subunits (α, β and γ). In their inactive state, a GDP molecule is bound to the α subunit. The interaction of an activated GPCR with the G protein induces the exchange of GDP to GTP, thus acting the receptor as a GEF (guanine nucleotide exchange factor). This switch produces the dissociation of the α subunit and the βγ dimers, leading to the subsequent modulation of different downstream effectors (Kostenis et al., 2005; Oldham and Hamm, 2008). The GTP bound to the active Gα is hydrolyzed by the intrinsic GTPase activity of this subunit. This process is accelerated by GTPase-activating proteins (GAPs). Gα-GDP re-associates with βγ dimers, re-assembling the heterotrimeric proteins and ending the G-protein activation cycle (Milligan and Kostenis, 2006)(figure 3).

G proteins are divided in four main classes based on amino acid identity of the Gα subunit: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Milligan and Kostenis, 2006). $G\alpha_s$ activates adenylyl cyclase (AC), increasing the levels of 3',5'-cyclic adenosine monophosphate (cAMP) and subsequently activating protein kinase A (PKA), which regulates

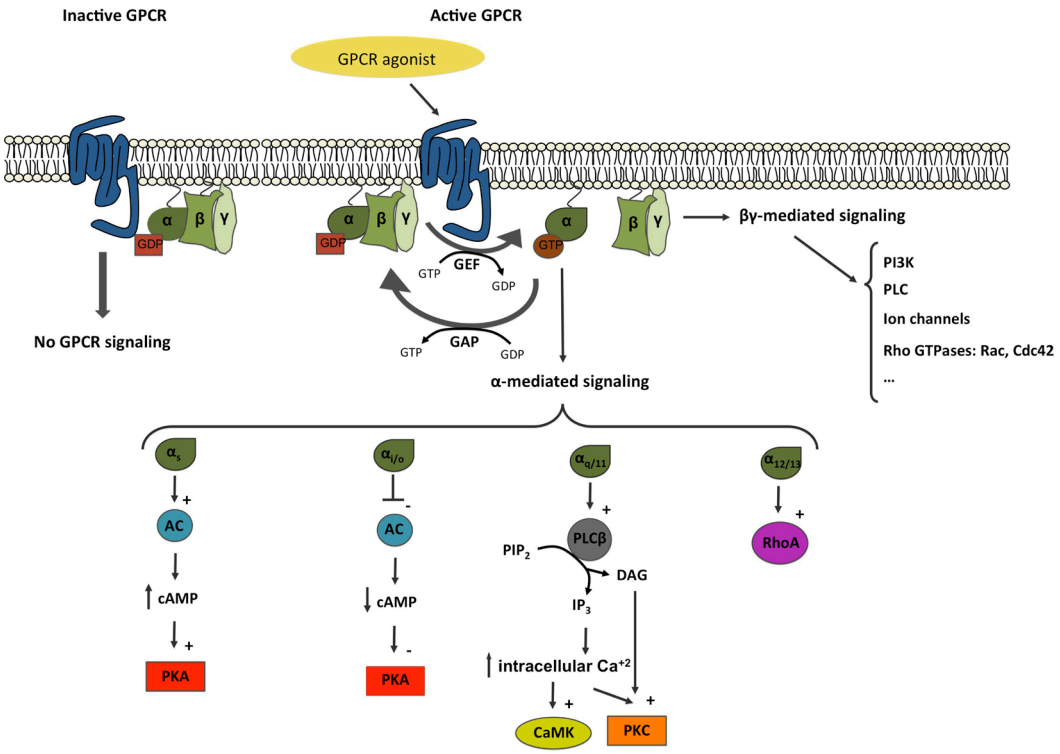

Figure 3. GPCR activation and signaling. *GPCR activation by ligand binding induces the interaction of the receptor with heterotrimeric G proteins. Both α and βγ and dimers activate then different signaling pathways that modulate a wide variety of biological responses. GEF, guanine nucleotide exchange factor; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GAP, GTPase-activating proteins; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; Cdc42, cell division control protein 42; AC, adenylate cylcase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PIP_2,phosphatidylinosito 4,5-biphosphate; IP_3, inositol-1,4,5-triphosphate; DAG, dyacilglycerol; CaMK, Ca^{2+}/ calmodulin-dependent protein kinase; PKC, protein kinase C; RhoA, Ras homolog gene family member A.*

many functions such as cell metabolism, cellular secretion and membrane permeability, among many others. On the contrary, $G\alpha_{i/o}$ inhibits AC, the production of cAMP and, for example, Ca^{2+} ion channels, leading to opposite effects. It has been shown that this pathway is particularly important in cell motility and regulation of neuronal transmission, for example. $G\alpha_{q/11}$ stimulates phospholipase C-β (PLCβ) that regulates the release of intracellular Ca^{2+} through the generation of IP_3 and DAG. This signaling participates in the control of the stimulation of mitogen-activated protein kinases (MAPKs) and the regulation of proliferation and production of cytokines, for example. $G\alpha_{12/13}$ activates mainly the Rho GTPase signaling pathway, which is important in cell proliferation, migration, regulation of cell-cell adhesion, and has been involved in many aspects of tumor progression (Marinissen and Gutkind, 2001; Milligan and Kostenis, 2006; Riobo and Manning, 2005; Wu et al., 2012)(figure 3).

Initially, it was believed that the βγ dimer was just binding to the Gα subunit, anchoring the heterotrimer to the plasma membrane and inhibiting spontaneous signaling. Today, it is well known that the βγ dimers can interact with and activate different effectors, such as ion channels, PLC and components of the MAPK cascades, producing specific functional responses (Cabrera-Vera, 2003; Milligan and Kostenis, 2006).

Non-G protein pathway

Although most GPCR-mediated effects are produced via signaling through heterotrimeric G proteins, it is currently admitted that GPCRs interact with many other proteins, collectively termed

GPCR-interacting proteins (GIPs). These interactions either modulate GPCR signaling or function to couple these receptors to heterotrimeric G protein-independent signaling pathways. In addition, GPCRs interact with proteins that regulate their trafficking to different cellular compartments, endocytosis or processing in the endoplasmic reticulum among others (Maurice et al., 2011; Pierce et al., 2002).

One of the most studied non-G protein signaling pathways is the one mediated by β-arrestins. β-arrestins are endocytic adaptor proteins that facilitate the targeting of receptors to clathrin-mediated endocytosis. They can bind to activated GPCRs that have been phosphorylated by G protein-coupled receptor kinases (GRKs) or second messenger protein kinases like PKA or PKC, thus directing the receptors to desensitation by endocytosis. β-arrestin binding is crucial for receptor desensitization and internalization. But β-arrestins can also act as adaptor proteins triggering heterotrimeric G protein-independent signaling. For example, β-arrestins can bind to the non-receptor tyrosine kinase Src family and can couple GPCRs to MAPK cascades [such as the extracellular signal-regulated kinase (ERK), p38 or c-Jun N-terminal kinase 3 (JNK3)], or to the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, among others (Luttrell and Lefkowitz, 2002; Maurice et al., 2011; Shukla et al., 2011).

GPCRs and cancer

GPCRs control crucial physiological functions, and their deregulation contributes to many pathologies like asthma, inflammation, obesity, pain, cardiovascular and central nervous system diseases and cancer. In fact, more than half of the therapeutic agents used in the clinic target directly or indirectly GPCRs (Dorsam and Gutkind, 2007; Wu et al., 2012).

Cancer is one of the most common diseases worldwide and one of the firsts causes of deaths in developed countries. Its incidence has grown in the past decades, and studies from the World Health Organization point to a continuous increase over the world in the coming years. Cancer is an evolutionary process that involves dynamic changes in the genome that result in accumulation of somatic mutations (Blanpain, 2013; Hanahan and Weinberg, 2000). During this process, normal cells acquire different capabilities that lead to malignant transformation. These capabilities are known as the “hallmarks of cancer”, and are defined by: 1) self sufficiency in growth signals, 2) insensitivity to anti-growth signals, 3) resistance to apoptosis, 4) sustained angiogenesis, 5) enhanced tissue invasion and metastasis capabilities and 6) limitless replicative potential (Hanahan and Weinberg, 2000; 2011).

Different studies have shown that GPCRs can modulate most, if not all, of these hallmarks (figure 4). The first direct link between cancer and GPCRs was reported in 1986, where the overexpression of the oncogene MAS (that encodes a GPCR) promoted focus formation *in vitro* and tumorigenesis *in vivo* (Young et al., 1986). Since then, aberrant GPCR signaling has been found in different cancers, favoring tumor development and progression (Li et al., 2005). This abnormal signaling is due either to aberrant overexpression of GPCRs (such as endothelin receptors, chemokine receptors, lysophosphatidic acid (LPA) receptors and sphingosine 1-phosphate (S1P) receptors), or to mutations in key residues that constitutively activate the receptor or by aberrant activation by their agonists, released by either tumor or stromal

cells (Dorsam and Gutkind, 2007; Li et al., 2005; O'Hayre et al., 2013).

GPCRs and uncontrolled cell proliferation

Normal cells have an extremely accurate machinery that controls their proliferation. They only divide in response to growth signals that bind to specific membrane receptors, activating different intracellular signaling cascades that promote cell cycle progression and proliferation. If these signals are not present, proliferation is inhibited. This tight control is crucial for the regulation of tissue homeostasis, structure and function. Cancer cells, by generating their own growth signals, can bypass these controls, which results in the persistent stimulation of their receptors and the consequent uncontrolled cell proliferation (Hanahan and Weinberg, 2000; 2011). Many potent mitogens such as thrombin, LPA, gastrin-releasing peptide (GRP), endothelin and prostaglandins stimulate cell proliferation by activating their specific GPCRs. Some of these receptors are overexpressed in cancer cells, promoting uncontrolled cell proliferation (Dorsam and Gutkind, 2007; Marinissen and Gutkind, 2001). This is the case, for example, of protease-activated receptor (PAR). When this receptor is activated by serine proteases such as thrombin, it couples to G_i, G_q and $G_{12/13}$ proteins, leading to the activation of diverse signaling pathways, including the pro-survival PI3K/Akt axis, MAPK cascades, Rho kinase and PLC, that promote cell survival, proliferation, migration and adhesion (Coughlin, 2000; Zigler et al., 2011). Another example of GPCRs overexpressed in cancer are the endothelin receptors (ET_A and ET_B). The vasocontrictive peptide endothelin, by binding to its specific receptor, stimulates the ERK/MAPK cascade, an important regulator of cell proliferation (Bagnato et al., 2011). Aberrant signaling mediated by overexpression of these receptors has been implicated in melanoma and

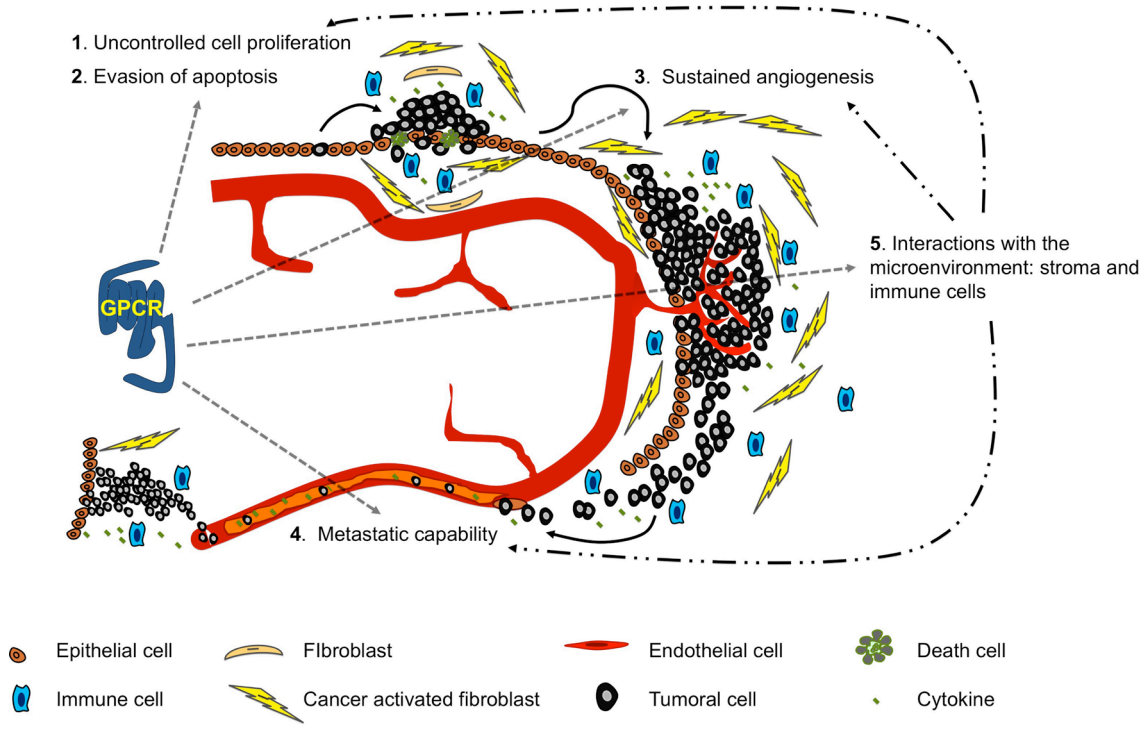

***Figure 4. Role of GPCRs in cancer development**. GPCRs promote cancer cell proliferation (1) and evasion of apoptosis (2), leading to tumor growth. They also promote sustained angiogenesis (3), enhance invasion and metastatic capabilities (4), and help tumor cells to create an advantageous tumor microenvironment that favors tumor progression (5).*

prostate cancer progression. Moreover, many patients with metastatic cancer present elevated levels of endothelin in plasma, which contributes to the overactivation of the system and the consequent worsening prognosis (Dorsam and Gutkind, 2007). LPA receptors are also overexpressed in many human tumors including lung, breast, stomach, ovary, kidney and prostate cancers (Murph, 2008; Panupinthu et al., 2010). LPA binds to 6 different LPA receptors [$LPA_{(1-6)}$] that are coupled to G_i, G_q and $G_{12/13}$ proteins, activating multiple oncogenic signaling pathways. In addition, elevated expression of autotaxin (the phospholipase D that produces LPA from lysophosphatidylcholine) and LPA are found in the plasma of certain cancer patients, increasing the aberrant signaling initiated by LPA (Panupinthu et al., 2010) (Box 1).

Interestingly, many orphan GPCRs are emerging as important molecular players in cancer, even though no specific ligand has been assigned to them yet. One example is GPR19. Overexpression of this receptor accelerates cell-cycle progression, conferring a proliferative advantage on lung cancer cells. This effect was associated with the modulation of cyclin B1 levels and the transition from G2 to mitosis in the cell cycle (Kastner et al., 2012).

GPCRs and resistance to apoptosis

When a normal cell detects genotoxic damage, antiproliferative or death-inducing signals or uncontrolled cell proliferation, it turns on antiproliferative signaling pathways and/or the death machinery. Cancer cells present the ability to evade these death signals, leading to cancer cell survival and the growth of tumors. Aberrant GPCR signaling has been implicated in this process. For example, S1P, by binding to its specific GPCRs, increases the expression of the anti-apoptotic protein BCL-2 (Sauer et al., 2005), inactivates the pro-apoptotic protein BAD and impairs caspase activation by blocking the translocation of BAX to the mitochondria (Betito and Cuvillier, 2006), thereby protecting the cells against apoptosis. Regarding orphan GPCRs, constitutive activation of GPR18, which is highly overexpressed in melanomas, is able to inhibit apoptosis by promoting cancer cell survival (Qin et al., 2011).

GPCRs and sustained angiogenesis

Once tumors reach a certain size in their primary site, they demand extra nutrients and oxygen supply that cannot be supported by the normal tissue vasculature. At this point, the tumors use different strategies to generate a new vascular network that will cover these highly demanding metabolic needs. The new blood vessels also provide a route of dissemination for cancer cells through the systemic circulation, allowing them to reach distant sites where they can generate new tumoral masses known as metastasis (Hanahan and Weinberg, 2000; 2011). To induce angioneogenesis, tumor cells promote survival, proliferation, migration and tube alignment of endothelial cells. The vascular endothelial growth factor (VEGF) is one of the main pro-angiogenic signals and it is upregulated in many tumors (Richard et al., 2001). Increased levels of VEGF often rely on thrombin overexpression. This serine protease activates PAR (GPCR) receptors, which stimulates the secretion of VEGF, increasing vascular permeability and inducing the production of matrix metalloproteinases (MMPs). These enzymes degrade the extracellular matrix, favoring the migration and proliferation of endothelial cells, and subsequent vessel formation (Richard et al., 2001; Yin et al., 2003). In addition, PAR-1 activa-

Box 1. Lysophospholipids and cancer. Lipids are the main component of cell membranes and essential sources of energy, but some of them are also important signaling mediators. Thus, they regulate cardiovascular functions, immune responses and brain development, just to name a few. Lysophospholipids are probably the best characterized signaling bioactive lipids, LPA (lysophosphatidic acid) and S1P (sphingosine 1-phsophato) being the most representative. They exert growth factor-like effects such as induction of proliferation, survival, migration, adhesion and differentiation (Mutoh and Chun, 2008). Both LPA and S1P mediate most of their biological functions by binding to and activating specific GPCRs. So far, 6 receptors have been identified for LPA (LPA_{1-3}, that belong to the endothelial differentiation gene (Edg) family and LPA_{4-6}, related to the purinergic receptor family) and 5 for S1P ($S1P_{1-5}$), but additional putative lysophospholipid receptors have been proposed in the literature (Mutoh et al., 2011).

Lysophopholipid metabolism and receptor activation are tightly regulated under normal physiological conditions. Their deregulation can lead or contribute to different pathologies, including cancer. Thus, ascitic fluid and plasma from patients with ovarian cancer present increased levels of LPA (Sutphen et al., 2004a; Xiao et al., 2001; Xu et al., 1995; 1998) and S1P (Hong et al., 1999). LPA is also increased in the plasma of hepatocellular carcinoma patients (Skill et al., 2012) and the levels of S1P in serum from stage IIIA human breast cancer patients is higher than that of healthy volunteers (Nagahashi et al., 2012). Moreover, LPA and S1P receptors are aberrantly expressed in tumors from different origins. Thus, LPA_2 and LPA_3 receptors are upregulated in ovarian (Fang et al., 2002), colon (Shida et al., 2004), thyroid (Schulte et al., 2001), breast (Kitayama et al., 2003), gastric (Yamashita et al., 2005a) and prostate (Im et al., 2000; Zeng et al., 2009) cancers. In addition, high expression of S1P receptors has been detected in thyroid (Balthasar et al., 2006), gastric (Yamashita et al., 2005b) and breast (Pyne, 2012) cancers. As well, the enzymes in charge of the synthesis of LPA and S1P are aberrantly expressed in tumors. LPA is produced extracellularly from lysophosphatidylcholine by autotaxin (ATX), a lysophospholipase D. ATX is overexpressed in tumors from different origins, such as breast cancer, glioblastoma, thyroid carcinoma, non-small cell lung cancer and hepatocellular carcinoma among others. Moreover, overexpression of ATX increases tumor growth, tumor aggressiveness and angiogenesis (Houben and Moolenaar, 2011; Murph and Mills, 2007). S1P is produced by sphingosine kinases (SKs) by catalizing the phosphorylation of sphingosine. The fact that SK1 overexpression induced the transformation of NIH3T3 fibroblasts, increasing cell growth and tumor development in immunodeficient mice, was one of the first evidences of the potential oncogenic role of SK1 (Xia et al., 2000). In addition, the expression of SK1 is elevated in many cancers such as breast, stomach, lung, colon or brain among others, and its expression corralates with increased tumor grade and reduce patient survival (Murph and Mills, 2007; Pyne and Pyne, 2010).

Both LPA and S1P receptor stimulation activates oncogenic pathways via $G\alpha_i$, $G\alpha_{12/13}$ and $G\alpha_q$, promoting cell survival, cell proliferation, cytoskeletal remodeling, cell migration and invasion among others, which in turn promotes tumor initiation and progression (Houben and Moolenaar, 2011; Pyne and Pyne, 2010).

tion induces the secretion of the angiogenic chemokines interleukin (IL)-8 and growth-regulated oncogene-α (GRO-α). These chemokines activate the chemokine receptors CXCR1 and CXCR2 on endothelial cells, inducing their proliferation, tube formation and migration, thus promoting angiogenesis (Agarwal et al., 2010). The chemokine CXCL12, by binding to and activating its specific GPCR (CXCR4), induces angiogenic responses as well in human basal cell carcinoma, breast cancer, pancreatic cancer and laryngeal and hypopharyngeal squamous cell carcinoma. Thus, tumors that express high levels of CXCR4 present higher blood vessel density as a consequence of

an enhanced secretion of angiogenic factors such as VEGF, IL-8 and IL-6 by tumor cells (Chu et al., 2009; Li et al., 2012; Liang et al., 2007; Matsuo et al., 2009).

Lysophospholipids and their GPCRs are also important mediators of angiogenesis. Both LPA and S1P are associated with endothelial cell proliferation, migration and survival (Morris et al., 2009). In line with this notion, the expression of $S1P_1$ receptors is enhanced in tumor vessels and their knocked-down *in vivo* suppresses tumor growth by inhibiting the stabilization of new blood vessels (Chae et al., 2004). Overexpression of LPA_1 and LPA_3 receptors in neuroblastoma cells enhanced the expression of *Vegf-A*, resulting in increased endothelial cells motility and angiogenesis promotion. A similar effect is produced by an active mutated form of LPA_1 (Kitayoshi et al., 2012). Of interest, the VEGF produced via activation of LPA receptors generates a positive feedback loop by inducing autotaxin (enzyme in charge of LPA generation) and LPA_1 expression (Ptaszynska et al., 2010).

GPCRs and enhanced tissue invasion and metastasic capabilities

The final stage in cancer is metastasis. Metastasis causes a significant reduction in patients' quality of life and is responsible for most cancer-related deaths. This process consists in the spreading of cancer cells from primary tumors to distant locations, and it is produced in four steps: i) cancer cells scape from the primary tumor and enter the blood or lymphatic vessels (intravasation); ii) these cancer cells have to survive in the circulation until they reach a new organ to colonize; iii) cancer cells leave the circulatory system (extravasation) and iv) they colonize the distant niche and generate a new tumor (Nguyen et al., 2009).

An important step in metastasis is degradation of the extracellular matrix, so the tumor cells can spread. This function is mediated in part by matrix metalloproteinases (MMPs) that promote cell invasion and motility. Expression and activity of MMP-2 and MMP-9 are frequently elevated in human cancer, which correlates with increased metastasis and poor prognosis (Hua et al., 2011). MMPs are regulated by activator protein-1 (AP1)-dependent transcription (Sato et al., 1993), a nuclear response that is elicit by most GPCRs (Dorsam and Gutkind, 2007). For example, S1P by activating $S1P_3$ receptors via ERK, p38 and Akt induces the expression and activation of MMP-9, which favors an invasion and migration phenotype (Kim et al., 2011).

In addition, activation of $G_{12/13}$ proteins by GPCRs has been associated to tumor migration and invasion. These G proteins activate effectors, such as Rho GTPases and cadherins among others, effectors that are essential in the control of cell-cell adhesion, organization of the actin cytoesqueleton, microtubules dynamics, etc., which in turn regulate cell invasion and metastasis (Kelly et al., 2007).

Certain types of cancers preferentially metastasize to specific organs. For example, most breast cancers generate metastases in the bones, lungs, brain and liver (Nguyen et al., 2009). Selectivity towards a specific organ is determined by different factors, including mechanical and anatomical considerations that will facilitate the settlement of the tumor cells in specific locations. This selectivity is also driven by factors that are expressed at the site of metastasis, either by tu-

mor cells or by stromal cells, and by intrinsic properties of the tumor cells themselves due to differential gene profiles (Ben-Baruch, 2007; Nguyen et al., 2009). It has been described that one of the main responsibles for organ-specific metastasis are chemokines and their GPCRs. Chemokines are released at the distant organs and the expression of their receptors by tumor cells directs their migration to these specific sites, regulating their adhesion and invasion capabilities (Ben-Baruch, 2007). Many breast tumors overexpress the chemokine receptor CXCR4, providing proliferative, pro-survival and pro-migratory advantages to cancer cells. The organs that are most frequently colonized by these cells are lymph nodes, lungs, bone marrow and liver. Interestingly, these organs express high levels of the CXCR4 endogenous ligand, the chemokine CXCL12, attracting the tumor cells to these sites (Dorsam and Gutkind, 2007; Fernandis et al., 2004; Hembruff and Cheng, 2009; Müller et al., 2001). Moreover, under hypoxia conditions, a factor of poor prognosis, the hypoxia-inducible factor-1 (HIF-1), is stabilized, activating genes that promote angiogenesis, anaerobic metabolism, cell survival and invasion (Harris, 2002). In different tumor cell lines, including breast cancer cells (Shim et al., 2006), glioblastoma cells (Zagzag et al., 2006), renal cell carcinomas (Staller et al., 2003), chondrosarcoma cells (Sun et al., 2010), oral squamous cell carcinomas (Ishikawa et al., 2009) and ileal carcinoid cells (Arvidsson et al., 2010), one of the genes activated by HIF-1 is CXCR4, which promotes cell invasion. Another example of a GPCR directing the metastatic spreading to a specific site is the C-C chemokine receptor type 7 (CCR7). This receptor is overexpressed in many human cancer cells such as breast cancer cells (Cabioglu et al., 2005; Müller et al., 2001), gastric cell carcinomas (Mashino et al., 2002), colorectal cell carcinomas (Günther et al., 2005), cervical cancer cells (Kodama et al., 2006), esophageal squamous cell carcinomas (Ding et al., 2003) and non-small lung cancer cells (Koizumi et al., 2007), conferring them migration and invasion properties, and specifying their metastasis to the lymph nodes. In support of this idea, metastatic lymph nodes present high levels of the CCR7 ligands (the chemokines CCL21 and CCL19) compared to healthy nodes (Müller et al., 2001; Wilson et al., 2006).

GPCRs and tumor microenvironment

Growing evidence indicates that the tumor microenvironment, made up of stromal and immune cells, plays a pivotal role in cancer progression. It was classically admitted that the immune cells present in tumors had the mission of eradicating the neoplastic lesion. However, emerging evidence suggest that these cells can also favor tumor formation and progression (Hanahan and Weinberg, 2011). Thus, many of the signals that induce cancer cell proliferation, angiogenesis and metastasis come not only from tumor cells but also from the immune system and stromal cells. Moreover, both immune and stromal cells produce prostaglandins, cytokines and/or chemokines that activate their cognate GPCRs, thereby favoring tumor progression (Dorsam and Gutkind, 2007). For example, the increased expression of IL-8/CXCR2 specifically in the tumor microenvironment enhanced colon cancer growth and metastasis, while selective deletion of CXCR2 inhibited cancer growth and angiogenesis (Lee et al., 2012). In addition, fibroblasts and pancreatic cancer cells co-operate to induce neoangiogenesis, by promoting endothelial cell proliferation, migration and tube formation (Matsuo et al., 2009). Fibroblasts produce the chemokine CXCL12 that induces both the proliferation and migration of

endothelial cells, and the invasion of tumor cells through activation of its receptor CXCR4 in a paracrine manner. Moreover, CXCL12 significantly enhanced the production in pancreatic carcinoma cells of IL-8, a chemokine that activates CXCR1 and CXCR2 receptors on endothelial cells, potentiating their proliferation, migration and tube formation (Matsuo et al., 2009). But the cooperation between fibroblasts and cancer cells is bidirectional as suggested by the fact that the production of CXCL12 in fibroblasts was increased by the co-culture with tumor cells. Together, these results indicate that the interaction between tumor and stroma cells promote tumor angiogenesis and metastasis by regulating the production of chemokines (Matsuo et al., 2009).

An emerging important component of tumor microenvironment are “CAFs”, named after cancer-activated fibroblasts, which are characterized by the expression of α-smooth muscle actin (α-SMA) and by the production of extracellular matrix proteins. Normal stroma in most organs contains minimal number of fibroblasts, while tumor stroma has an increased number of these cells (Kalluri and Zeisberg, 2006). CAFs from human breast carcinomas secrete high levels of CXCL12, that by activating CXCR4 receptors, promote the growth of breast carcinoma cells. Additionally, CXCL12 mediates the chemotaxis and recruitment of endothelial progenitor cells into the carcinoma, inducing angiogenesis (Orimo et al., 2005).

Tumor-associated macrophages interact with tumor cells as well, promoting tumorigenesis and metastasis (Hao et al., 2012). Some GPCRs play essential roles in this interaction. For example, macrophages that express the endothelin receptor ET_B are directed towards hypoxic tumor areas, which express elevated levels of endothelins (Grimshaw et al., 2002). As a validation of this observation, selective knock-down of the ET_B receptor in the stroma reduced the number of infiltrating macrophages and, as a consequence, diminished breast cancer tumor growth and metastasis (Binder et al., 2009).

2. GPR55

The wide spectrum of action of GPCRs in cancer physiopathology makes them potential antitumoral targets. However, only few a GPCRs are currently being therapeutically exploited in oncology. Understanding the mechanism of action of GPCRs in cancer initiation and progression is therefore crucial to increase the therapeutic armamentarium based on these receptors. Orphan GPCRs, with no endogenous ligand assigned yet and whose physiological functions are in most cases unknown, constitute a very attractive population for such studies.

The orphan receptor GPR55 was first identified and cloned in 1999 (Sawzdargo et al., 1999). GPR55 belongs to the rhodopsin-like family of GPCRs, and its amino acid sequence share significant identities with the LPA recptors. Thus, GPR55 has 30% sequence identity with LPA_5 and LPA_4, and 29% with LPA_6 (Elbegdorj et al., 2013; Sawzdargo et al., 1999).

GPR55 pharmacology

According to the International Union of Basic and Clinical Pharmacology (IUPHAR), GPR55 is still an ophan GPCR. However, several reports suggest that L-α-lysophosphatidylinositol (LPI) is a potential endogenous receptor ligand. Thus, Oka and coworkers reported that GPR55 is engaged

by LPI in a model of ectopic overexpression of the receptor in HEK293 cells (Oka et al., 2007). This observation was corroborated by other reports in different cell types (Henstridge et al., 2009; Kapur et al., 2009; Lauckner et al., 2008; Oka et al., 2010; 2009; Whyte et al., 2009; Yin et al., 2009). Specifically, 2-arachidonoyl-LPI species have been described as the most efficient and potent in activating GPR55 (Oka et al., 2009).

But LPI is not the only ligand paired with GPR55. Few years after its identification and cloning, two different patents described the receptor as a putative cannabinoid receptor (Baker et al., 2006). Cannabinoids were originally described as the active compounds of the plant *Cannabis sativa*, but nowadays the term cannabinoids also includes the endogenous compounds produced by animals -the so called endocannabinoids- and synthetic molecules (Pertwee et al., 2010a). Cannabinoids mediate most of their effects by binding to and activating specific GPCRs. So far, two bona-fide cannabinoid receptors have been cloned, CB_1 (highly expressed in the central nervous system) (Matsuda et al., 1990) and CB_2 (mostly expressed in the immune system) (Munro et al., 1993). Different evidences have suggested that other non-CB_1/non-CB_2 cannabinoid receptor exist, being GPR55 one of the candidates (Mackie and Stella, 2006; Pertwee et al., 2010b; Ross, 2009). First, a patent from GlaxoSmithKline described the activation of GPR55 in yeast by AM251 and SR141716A, two well-established CB_1 receptor antagonists. A second patent from AstraZeneca described that the synthetic cannabinoid CP-55940, the endocannabinoids anandamide (AEA), 2-arachidonoylglycerol (2-AG), the marijuana-derived cannabinoid Δ^9-tetrahydrocannabinol (THC) and the cannabinoid-related compounds virodhamina and palmitoylethanolamide (PEA) were potent agonists on GPR55 in a model of membranes derived from HEK293 cells (Baker et al., 2006). However, whether GPR55 can be considered a cannabinoid receptor is still a controversial issue (Ross, 2009; Sharir and Abood, 2010). First, GPR55 shares low amino acid sequence identity with CB_1 (13.5%) and CB_2 (14.4%) (Box 2), and second, and most importantly, there is no consistency among studies on whether and which cannabinoids activate GPR55. For example, while some studies have shown that AEA and

Box 2. GPR55 structural peculiarities and divergences with CB_1 and CB_2 receptor. The structure of GPR55 contains most of the highly conserved residues of the class A GPCRs and cannabinoid receptors, but presents some interesting peculiarities. For example, the binding pocket of GPR55 is lined with hydrophilic residues almost to the intracellular side of the receptor, with a Lys80 residue essential for GPR55 ligand recognition (Elbegdorj et al., 2013). In contrast, CB_1 and CB_2 receptors present highly hydrophobic binding pockets (Gasperi et al., 2013; Kotsikorou et al., 2011). Another relevant difference is in the TM7 region. Like LPA_4, LPA_5 and LPA_6 receptors, GPR55 lacks the highly conserved NPXXY motif (present in CB_1 and CB_2 receptors), and has a DVXXY sequence instead that produces different hydration and local transmembrane flexibility, and in the end, a different conformation in this region (Kotsikorou et al., 2011). It has been described that class A GPCRs present a cholesterol binding site defined by a higly conserved consensus motif (CCM). Sequence analysis showed that GPR55 and CB_1, in contrast with CB_2, do not contain CCM, suggesting that these receptors might contain other motifs responsible for interaction with cholesterol and lipid rafts. On the other hand, cholesterol recognition amino acid consensus motifs (CRAC) are presented and located in GPR55 in the same region as in CB_1 and CB_2 receptors, [i.e., at the level of TM7 (Gasperi et al., 2013)]. All these differences may explain, at least in part, the different ligand activation and biological activity of GPR55, CB_1 and CB_2 receptors.

2-AG behave as GPR55 agonists (Lauckner et al., 2008; Ryberg et al., 2007; Waldeck-Weiermair et al., 2008), other authors did not find any effect of these compounds via GPR55 (Kapur et al., 2009; Oka et al., 2007). The same occurs with other cannabinoids, such as THC, CP-55940 or JWH-015 (Sharir and Abood, 2010). In this controversial pharmacological scenario, it is widely accepted however that the synthetic CB_1 antagonists AM251 and SR141716A are GPR55 agonists (Balenga et al., 2011; Henstridge et al., 2009; Kapur et al., 2009; Kargl et al., 2012; Kotsikorou et al., 2011), and that cannabidiol (CBD), a plant-derived cannabinoid with very low affinity for classical cannabinoid receptors, is a GPR55 antagonist (Balenga et al., 2011; Ford et al., 2010; Ryberg et al., 2007; Whyte et al., 2009).

The inconsistences on GPR55 pharmacology may rely on tissue, cell type and context, but they could also be explained by the specific characteristics of GPCRs. First, it has been shown that GPCRs can be in different active conformation states and that ligands present different affinity for these conformational states, which lead to the activation of different intracellular signaling pathways (Park, 2012). Another possible explanation is the "biased agonism", by which certain ligands can trigger non-G protein signaling pathways, activating only a subset of effectors (Audet and Bouvier, 2012; Venkatakrishnan et al., 2013). In addition, it has been described that GPCRs can form oligomers that modulate their individual functions, activating different and specific signaling pathways (Audet and Bouvier, 2012; Ferré et al., 2009). In line with this idea, a functional interaction between

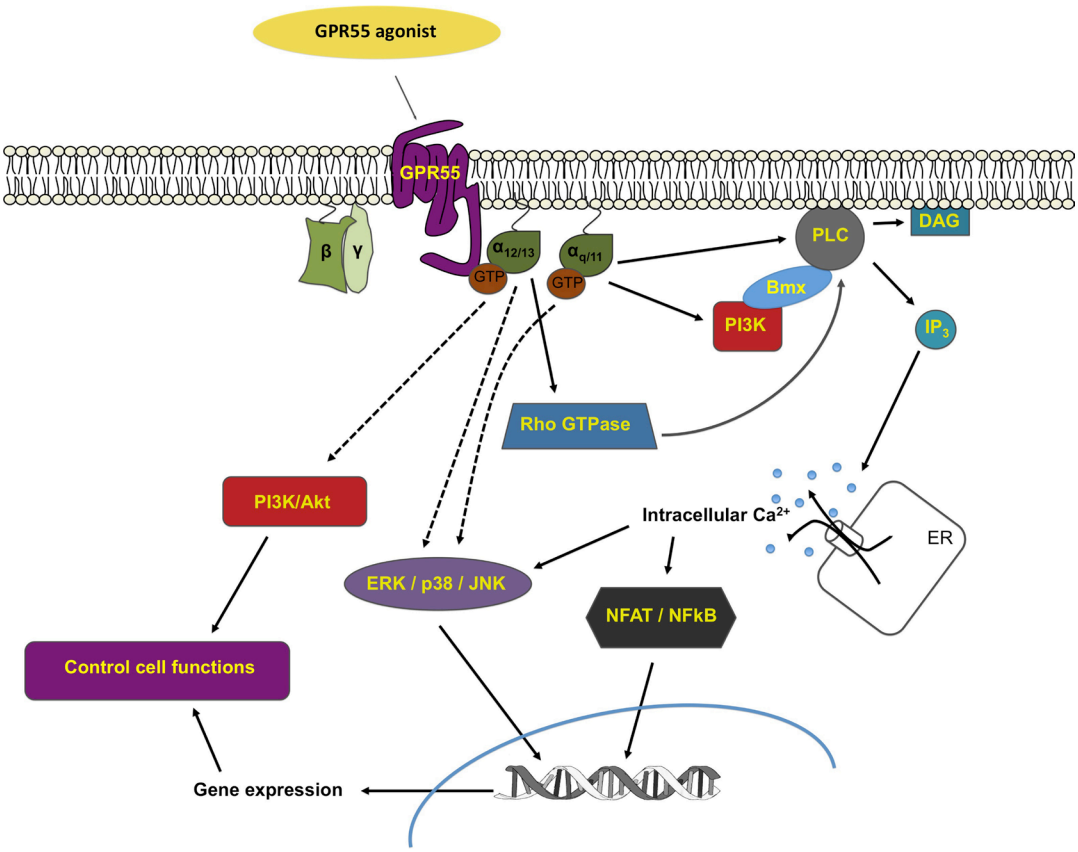

Figure 5. Signaling pathaways activated by GPR55. *GTP, guanosine 5'-triphosphate ; PI3K, phosphatidylinositol 3-kinase ; Bmx, bone marrow kinase X; PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; NFAT, nuclear factor of activated T-cells; NFkB, nuclear factor kappa-light-chain-enchancer of activated B cells; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.*

GPR55 and CB_2 receptor has been described in neutrophils (Balenga et al., 2011), and an interaction between CB_1 receptors and GPR55 has been reported in a HEK293-based model (Kargl et al., 2012). Kargl and coworkers showed that the activation of ERK, nuclear factor of activated T-cells (NFAT) and serum-response element (SRE) induced by activation of GPR55 by SR141716A and GSK319197A (a proposed GPR55 synthetic agonist) is not evident in the presence of an inactive CB_1 receptor. On the contrary, if both receptors are activated, CB_1 internalizes and GPR55 is able to signal (Kargl et al., 2012).

GPR55 signaling pathways

The mechanism by which GPR55 transforms an external stimulus into a biological response has been studied in different cellular models such as endothelial cells, osteoclasts, large dorsal root ganglion or tumoral cells. All the studies conclude that, while CB_1 and CB_2 signal mostly via $G_{i/o}$, GPR55 couples preferentialy to $G_{12/13}$ [with the subsequent activation of RhoA (Balenga et al., 2011; Brown et al., 2011; Henstridge et al., 2009; Huang et al., 2011; Lauckner et al., 2008; Obara et al., 2011; Oka et al., 2010; Ryberg et al., 2007)] and/or G_q [producing the activation of PLC (Lauckner et al., 2008; Obara et al., 2011; Waldeck-Weiermair et al., 2008)].

By engaging either G protein, GPR55 activates different signalling pathways, including MAPK cascades, cytoplamsic Ca^{2+} increase or actin filament formation. In HEK293 cells, Henstridge and coworkers described that the activation of GPR55 induces an oscillatory Ca^{2+} release from intracellular stores via $G_{12/13}$/RhoA/ROCK, with the subsequent phosphorylation and activation of the ERK/MAPK cascade and activation of transcription factors such as NFAT or nuclear factor kappa-light-chain-enchancer of activated B cells (NFkB), that translocate to the nucleus and modulate the expression of different genes (Henstridge et al., 2009; 2010). Other authors have described similar pathways in other cell types like dorsal root ganglia neurons (Lauckner et al., 2008), neutrophils (Balenga et al., 2011) or osteoclasts (Whyte et al., 2009). Other studies have revealed that GPR55, also via $G_{12/13}$, is able to activate the p38/MAPK cascade and the activating transcription factor 2 (ATF-2) in IM9 lymphoblastoid cells (Oka et al., 2010), and to istimulate JNK in cholangiocarcinoma calls (Huang et al., 2011) (figure 5).

Coupling of GPR55 to G_q modulates alternative signalling pathways. Thus, GPR55 activates PLC, releasing IP_3 (with the subsequent increase of intracellular Ca^{2+}), and DAG (in charge of activating PKC and different MAPKs) (Lauckner et al., 2008; Obara et al., 2011). In endothelial cells, Waldeck-Weiermair and coworkers described that GPR55, via G_q, triggers the PI3K-bone marrow kinase X-linked (Bmx)-PLC cascade, with IP_3 production and intracellular Ca^{2+} mobilization (Waldeck-Weiermair et al., 2008).

GPR55 physio-pathology

GPR55 is widely expressed throughout the body. Its mRNA and/or protein have been found in different regions of the brain such as caudate, putamen, frontal cortex, striatum and hypothalamus (Sawzdargo et al., 1999), as well as in glial cells (Pietr et al., 2009) and large dorsal root ganglia neurons (Lauckner et al., 2008). GPR55 is also expressed in peripheral tissues including spleen, adrenal glands, gastrointestinal tract (Ryberg et al., 2007; Sawzdargo et al., 1999), bone (Whyte et al., 2009), liver (Huang et al., 2011), pancreas

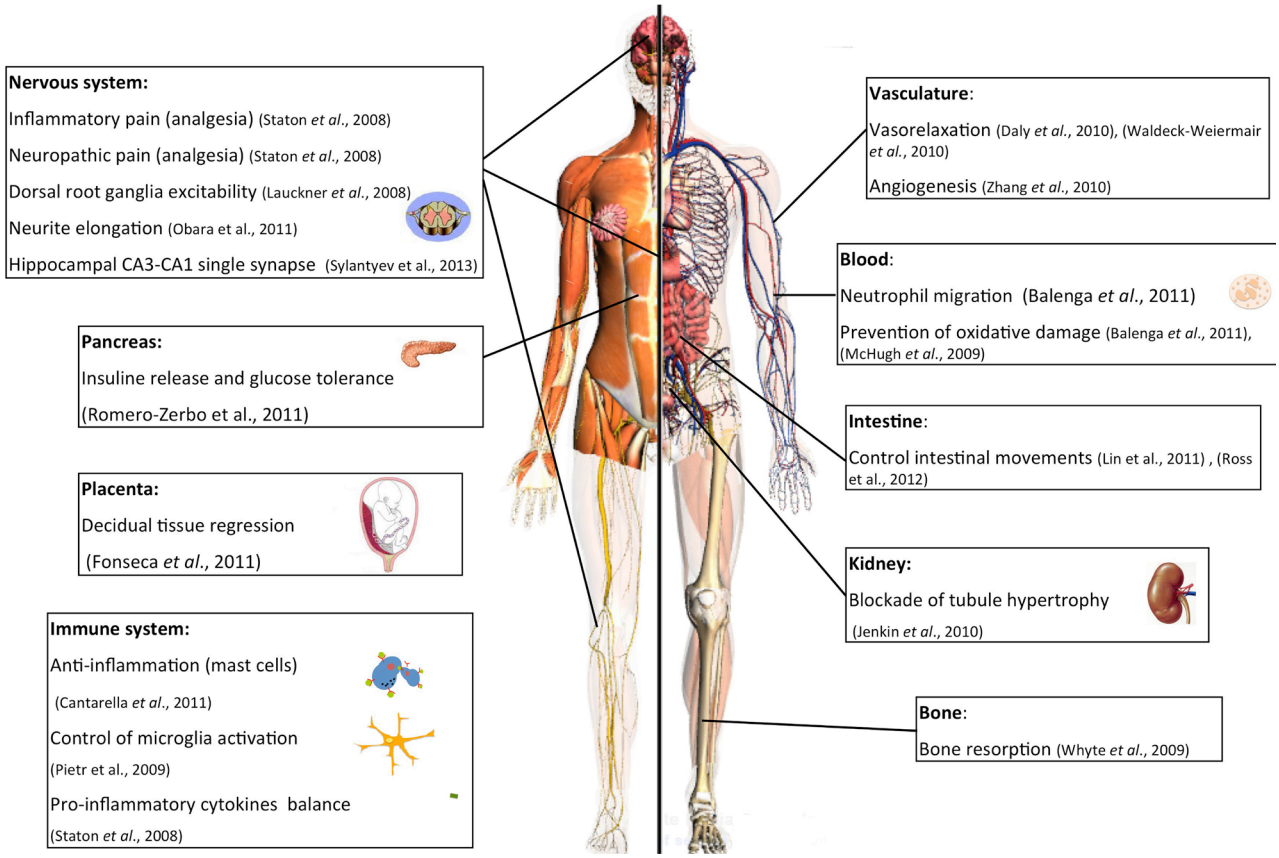

Figure 6. Patho-physiological relevance of GPR55. *In the mouse nervous system, GPR55 regulates dorsal root ganglia excitability, neurite elongation, hippocampal CA3-CA1 single synapse, controls inflammatory and neuropathic pain and microglia activation. In blood, GPR55 regulates neutrophil migration and may prevent oxidative damage. GPR55 is also involved in bone metabolism, inducing bone resorption. Other studies have suggested additional roles for GPR55 in modulating vascular function [by inducing vasorelaxation and controlling angiogenesis, renal tubule hypertrophy, decidual tissue regression during pregnancy, and mast cell-mediated anti-inflammatory actions]. GPR55 also controls intestinal movements and energy metabolism by regulating insulin release and glucose tolerance by the langerhans islets. Figure adapted from (Henstridge et al., 2011).*

(Romero-Zerbo et al., 2011), kidney (Jenkin et al., 2010), endothelial cells (Daly et al., 2010; Waldeck-Weiermair et al., 2008; Zhang et al., 2010), vascular smooth muscle cells (Daly et al., 2010; Fonseca et al., 2011), neutrophils (Balenga et al., 2011) and mastocytes (Cantarella et al., 2011a), suggesting that it may be involved in the control of many different biological functions (figure 6).

GPR55 has been implicated in the control of pain. Thus, activation of this receptor enhances neuronal excitability by increasing intracellular Ca^{2+} and suppressing the K^{+} current acting on M-type channels (Lauckner et al., 2008). These data point to a pro-nociceptive role of GPR55. In line with this idea, GPR55 deficient mice, unlike their WT counterparts, do not show hyperalgesia upon inflammatory or neuropathic stimuli (Staton et al., 2008). This was related to an abnormal increase of cytokines like IL-4, IL-10, interferon (IFN)-γ and granulocyte macrophage-colony stimulating factor (GM-CSF) in GPR55 KO mice after noxious stimulation. The role of GPR55 in inflammation is further supported by studies performed in microglial cells. For example, GPR55 expression changes during primary microglia cell activation. Lipopolysaccharide (LPS) or IFN-γ treatment induce a significant downregulation of GPR55 mRNA levels in primary microglia, while both activators induce GPR55 overexpression in an immortalized

and highly proliferative cell line (BV-2) (Pietr et al., 2009).

These reports suggest an involvement of GPR55 in inflammation within the central nervous system (CNS). But a role for GPR55 in the immune response in the periphery has also been described. GPR55 is expressed in neutrophils, where it functionally interacts with CB_2, also expressed in these cells (Balenga et al., 2011). Activation of both receptors produces a cooperative induction of neutrophil chemotaxis, involving the synergic activation of RhoA and Cdc42, which results in an enhanced migratory response to the inflammatory foci (Balenga et al., 2011). In addition, GPR55 regulates the excessive generation of reactive oxygen species produced by CB_2 agonists in these cells, controlling CB_2-mediated oxidative damage (Balenga et al., 2011). The expression of GPR55 has also been reported in mastocytes, where it seems to play an anti-inflammatory role. Activation of GPR55 in these cells blocks the release of nerve growth factor (NGF), a factor induced by inflammatory agents (Cantarella et al., 2011a). A recent study described GPR55 expression in uterine natural killer cells, suggesting an involvement of GPR55 in the immunological reactions during pregnancy. The authors also observed that activation of GPR55 in these cells induces decidual cell death, suggesting a role of GPR55 in fetoplacental development (Fonseca et al., 2011).

Although GPR55 is expressed in the CNS, the lack of the receptor does not alter gross brain structures or CNS development (Wu et al., 2013). Thus, general behavioural responses, gross motor skills, sensory-motor integration, learning and memory or anxiety and depressive behaviors are not modified in GPR55 KO mice. However, in more challenging motor responses, GPR55 deficient mice present impaired movement coordination (Wu et al., 2013). Supporting the involvement of GPR55 in the control of central functions, it has been shown that GPR55 regulates neurite elongation. The authors reported that LPI, via GPR55-$G_{12/13}$-RhoA, produces neurite retraction, accompanied by loss of light chain neurofilaments and redistribution of F-actin in differentiated cells. This effect was accompanied by the localization of the receptor in growth cones or ruffled borders in differenciated neurons (Obara et al., 2011). A recent study has described the involvement of GPR55 in synaptic circuits, specifically in hippocampal single CA3-CA1 synapses (Sylantyev et al., 2013). GPR55 activation induces intracellular Ca^{2+} release from presynaptic stores that prompts a transient increase in release probability at CA3-CA1 synapses (Sylantyev et al., 2013).

GPR55 has also been implicated in bone physiology. *In vitro* activation of GPR55 by LPI stimulates osteoclast polarization and resorption activity. A similar effect was observed *in vivo*, where male GPR55 KO mice presenting a decreased bone resorption associated with high bone mass and an osteopetrotic phenotype (Whyte et al., 2009).

Recent studies propose that GPR55 controls intestinal movements (Lin et al., 2011; Ross et al., 2012) and energy metabolism. GPR55 is highly expressed in pancreatic islets and insulin-secreting β-cells. GPR55 activation in Langerhans islets *in vitro* produces an increase in intracellular Ca^{2+} and glucose-dependent insulin secretion. Moreover, *in vivo* activation of GPR55 enhances glucose tolerance and the levels of insulin in plasma (Romero-Zerbo et al., 2011). Interestingly, high levels of GPR55 are associated with visceral adiposity and type-2 diabetes, and obese patients

present higher circulating levels of LPI than lean individuals (Moreno-Navarrete et al., 2012).

It has been suggested that GPR55 may regulate vascular functions as well. Thus, GPR55 is expressed by vascular endothelial cells, and these cells produce LPI, the putative GPR55 endogenous ligand (Bondarenko et al., 2010; Brown, 2007; Daly et al., 2010; Johns et al., 2007; Ryberg et al., 2007; Waldeck-Weiermair et al., 2008). However, although many reports have studied the role of GPR55 in the vasculature, there are not convincing results establishing a direct link between the two elements. Many studies have reported the presence of a non-CB_1/non-CB_2 cannabinoid-sensitive vascular receptor that could be regulating the vasodilating effects of some cannabinoids such as AEA and abnormal cannabidiol (abn-CBD)(Kreitzer and Stella, 2009). The presence of GPR55 in endothelial cells and its activation by AEA and abn-CBD (Ryberg et al., 2007) point to this receptor as a likely candidate to constitute the non-CB_1/non-CB_2 receptor in the vasculature that mediates those effects. However, blood pressure and heart rate were similar in GPR55 KO and WT mice. Moreover, there were no differences in the vascular relaxation induced by abn-CBD in GPR55 KO and WT mice (Johns et al., 2007), suggesting that, although GPR55 is present in the vasculature, it is not the site of action of abn-CBD. GPR55 signaling in endothelial cells might be controlled by the cellular context. Thus, Waldeck-Weiermair and coworkers have suggested a possible downstream interaction between GPR55 and CB_1 in endothelial cells, conditioned by this cellular context. In this case, depending on the clustering state of the integrins, AEA, a ligand for both receptors, activates one receptor, inhibiting the other, or *vice versa* (Waldeck-Weiermair et al., 2008). A recent report, however, confirms a role for GPR55 in vascular functions, specifically in angiogenesis. Thus, activation of the receptor stimulates tube formation, endothelial cell migration and upregulation of the angiogenesis inducing signal VEGF (Zhang et al., 2010).

Different indirect evidences suggest that GPR55 could be involved in cancer physio-pathology. First, increased levels of LPI, the putative GPR55 endogenous ligand, have been found in plasma and ascites from patients with ovarian cancer compared with women without oncogenic pathologies (Sutphen et al., 2004b; Xiao et al., 2001). Moreover, epithelial cells (Falasca and Corda, 1994) and fibroblasts (Falasca et al., 1998) are able to generate mitogenic LPI after Ras induced transformation. Second, GPR55 couples to $G_{12/13}$ and G_q, which are known to have oncogenic properties (Dorsam and Gutkind, 2007; Wu et al., 2012). For example, overexpression of the $G_{12/13}$ stimulates mitogenic signals that induce fibroblast transformation (Dhanasekaran and Dermott, 1996). Moreover, GPR55 signals through Rho GTPases, which control cytoskeleton organization, cell polarity and cell migration, all of them intimatelly related to tumor progression (Karlsson et al., 2009). Finally, certain cannabinoids -compounds with known antitumoral actions (Velasco et al., 2012)- can activate GPR55 (Sharir and Abood, 2010), suggesting that this receptor may be part of a system that controls tumor generation and/or progression.

AIMS OF THIS THESIS

Since the orphan G protein-coupled receptor GPR55 was first cloned in 1999, different laboratories started to analyze the physiological relevance and mechanism of action of this receptor. Although almost 15 years later little is still known about these two issues, it has been proposed that GPR55 regulates responses in the central nervous system, bone, immune system and vasculature, among others, and that most GPR55-mediated effects are produced via coupling to G_q and $G_{12/13}$ proteins. Considering that (a) some GPCRs are associated to cancer, (b) signaling properties of GPR55 and (c) that lysophospholipids and cannabinoids (two putative families of GPR55 ligands) play important roles in cancer, the working HYPOTHESIS of this Thesis is that GPR55 participates in the control of tumor physio-pathology.

In this context, the spefic AIMS of this study are:

1. To study the expression of GPR55 in human tumors.

2. To investigate the role of GPR55 in tumor generation and progression.

3. To analyze whether GPR55 is involved in the effect of cannabinoids on cancer cell proliferation.

RESULTS AND DISCUSSION

CHAPTER 1

In this first chapter we will try to determine whether GPR55 plays any significant role in cancer physiopathology. First, we will analyze the expression of GPR55 in a collection of human tumor cell lines and human breast cancer, glioblastoma and pancreatic adenocarcinoma samples. In addition, we will modulate GPR55 expression in cancer cell lines (by transfection with HA-GPR55 constructs or genetic knock-down with specific siRNAs) to determine the involvement of GPR55 in cancer cell proliferation both *in vitro* and *in vivo*. We will also try to characterize the molecular mechanisms underlying GPR55 action on cancer cells.

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SHORT COMMUNICATION

The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK

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GPR55 is an orphan G protein-coupled receptor that may be engaged by some lipid ligands such as lysophosphatidylinositol and cannabinoid-type compounds. Very little is known about its expression pattern and physio-pathological relevance, and its pharmacology and signaling are still rather controversial. Here we analyzed the expression and function of GPR55 in cancer cells. Our data show that GPR55 expression in human tumors from different origins correlates with their aggressiveness. Moreover, GPR55 promotes cancer cell proliferation, both in cell cultures and in xenografted mice, through the overactivation of the extracellular signal-regulated kinase cascade. These findings reveal the importance of GPR55 in human cancer, and suggest that it could constitute a new biomarker and therapeutic target in oncology.
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Keywords: G protein-coupled receptors; GPR55; cancer; cannabinoids

G protein-coupled receptors (GPCRs) constitute the largest superfamily of cellular receptors. They control crucial physiological functions and, consequently, their dysfunction contributes to many human diseases. In fact, they are—together with enzymes—the most common target of therapeutic drugs (Dorsam and Gutkind, 2007). Deorphanizing GPCRs and expanding our knowledge on GPCR-mediated signaling pathways is therefore a pivotal strategy to design new diagnostic and therapeutic tools for human pathologies. It has been recently proposed that the orphan GPCR GPR55 is engaged and activated by lysophosphatidylinositol (LPI) (Oka *et al*., 2007). This original observation, made in cells ectopically expressing GPR55, was shortly corroborated by other reports (Lauckner *et al*., 2008; Henstridge *et al*., 2009; Kapur *et al*., 2009; Oka *et al*., 2009, 2010; Yin *et al*., 2009). Moreover, LPI has been found to activate GPR55 in cells in which the receptor is endogenously expressed (large dorsal root ganglion neurons (Lauckner *et al*., 2008), osteoclasts (Whyte *et al*., 2009) and lymphoblastoid cells (Oka *et al*., 2010)), supporting the notion that this phospholipid may be an endogenous GPR55 ligand. Nonetheless, all the functions described so far for LPI on GPR55 come from experiments in which LPI was exogenously added to the cultured cells, and therefore evidence for the role of the naturally occurring lipid in more physiological settings is still missing. It has also been shown that several cannabinoid-type compounds modulate this receptor (Ryberg *et al*., 2007; Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008; Henstridge *et al*., 2009, 2010; Kapur *et al*., 2009; Yin *et al*., 2009). However, the inconsistencies among the pharmacological results obtained so far (some compounds being active in some reports and inactive in others, some being agonists in some studies and antagonists in others, and so on) do not entirely clarify whether GPR55 is an actual cannabinoid receptor (Brown and Robin Hiley, 2009; Ross, 2009). GPR55 mRNA is highly expressed in the brain, the adrenal glands, parts of the gastrointestinal tract, spleen, tonsils, testes, thymus (Sawzdargo *et al*., 1999; Ryberg *et al*., 2007; Oka *et al*., 2010), large dorsal root ganglion neurons (Lauckner *et al*., 2008), osteoclasts (Whyte *et al*., 2009), certain microglial cells (Pietr *et al*., 2009), endothelial cells and mesenteric arterial smooth muscle cells (Daly *et al*., 2010), but very little is known about the physiological role of the receptor in these or other tissues. To date, GPR55 has been implicated in the control of pain, specifically in the mechanical hyperalgesia induced by inflammatory and neuropathic pain (Staton *et al*., 2008), and in the control of bone formation (Whyte *et al*., 2009). Its wide distribution throughout the body suggests, however, that GPR55 might be involved in many other biological functions. As some GPCRs have a prominent role in cancer cell biology (Dorsam and Gutkind, 2007) and, more specifically, GPR55 has been shown to couple to $G_{12/13}$ and G_q proteins (Ryberg *et al*., 2007; Lauckner *et al*., 2008; Henstridge *et al*., 2009), which drive oncogenic signaling (Dorsam and Gutkind, 2007; Worzfeld *et al*., 2008), we sought to analyze the physiopathological relevance of this receptor in the context of cancer.

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Supplemental Table 1

Cell line	Origin	GPR55 expression	Cell line	Origin	GPR55 expression
A172	Glioblastoma	+	BT-474	Breast ductal carcinoma	+
CCF-STTG1	Astrocytoma	++	EVSA-T	Breast adenocarcinoma	++
GOS-3	Astrocytoma/ Oligodendroglioma	++	MCF-7	Breast adenocarcinoma	+
H4	Neuroglioma	+	MDA-MB-231	Breast adenocarcinoma	+
LN-405	Astrocytoma	++	MDA-MB-468	Breast adenocarcinoma	+
SW 1088	Astrocytoma	+	SK-BR-3	Breast adenocarcinoma	-
SW 1783	Astrocytoma	+	T-47D	Breast ductal carcinoma	-
T98G	Astrocytoma	++	A-375	Melanoma	++
U-87 MG	Astrocytoma	+	HeLa	Cervix adenocarcinoma	+
U-118 MG	Astrocytoma	+	Hep-G2	Hepatocellular carcinoma	+
U-373 MG	Astrocytoma	+	JURKAT	T cell leukemia	+
			IM-9	B lymphoblastoid myeloma	++
			MIA PaCa-2	Pancreas carcinoma	+

Table S1. Human cancer cell lines express GPR55 mRNA. Human cell lines were obtained from The American Type Culture Collection (Manassas, VA) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). RNA was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA), including a DNase digestion step, with the Real Star Kit (Durviz, Valencia, Spain), and cDNA was obtained with Transcriptor Reverse Transcriptase (Roche, Basel, Switzerland). The primers used for real time-quantitative PCR for human GPR55 were: sense 5'-CTGCCTTGGTTCCACCATA-3' and antisense 5'-CCAGGATGCAGGTGAGTAAGA-3'. The probe was from the Universal Probe Library (Roche). 18S RNA was used as reference.

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Supplemental Figure 1

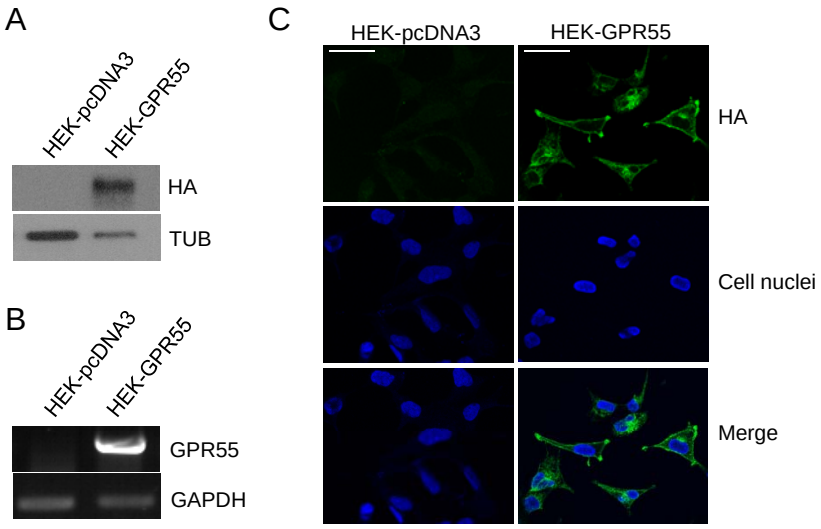

Figure S1. Characterization of GPR55-overexpressing HEK293 cells. HEK293 cells stably expressing 3xHA-GPR55 were previously generated (Henstridge *et al.*, 2009). (A) Western blot analysis of HA-tagged GPR55. Antibodies were: anti-HA (Cell Signaling Technology, Danvers, MA) and anti-α-tubulin (used as loading control, Sigma-Aldrich, St. Louis, MO). (B) GPR55 mRNA expression as determined by RT-PCR. Primers were: sense 5'- GTCCCCCTTCCCGTCCCTGTG-3' and antisense 5'- GCTGGCTGCGATGCTGTAGATGC-3'. GAPDH was used as internal control. (C) Immunofluorescence analysis of GPR55 expression by anti-HA staining (in green). Cell nuclei (in blue) were stained with Hoechst 33342 (Invitrogen, Carlsbad, CA). Scale bar, 4µm.

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Supplemental Figure 2

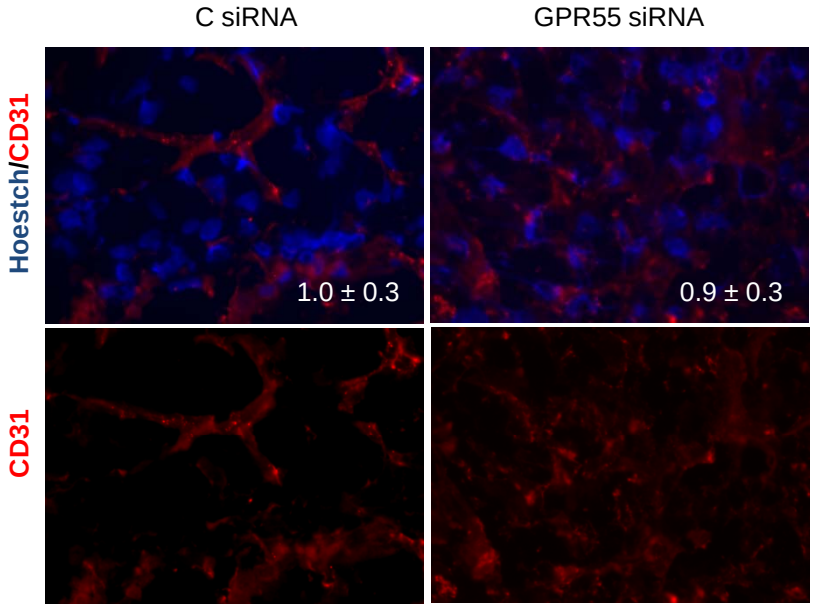

Figure S2. Immunofluorescence analysis of CD31 staining in T98 cells-derived xenografts. The potential antiangiogenic effect of GPR55 silencing *in vivo* was determined by immunofluorescence analysis of the vascular endothelial marker CD31. Tissue-tek embedded paraformaldehyde fixed tumor sections were incubated with anti-CD31 antibody (Pharmingen/BD Biosciences, San Jose, CA). The secondary anti-rabbit Alexa Fluor 594 antibody was from Invitrogen (Carlsbad, CA). Cell nuclei were stained with Hoechst 33342 (Invitrogen) and are shown in blue. Confocal fluorescence images were acquired using Laser Sharp 2000 software (Bio-Rad) and CD31 staining intensity (expressed in arbitrary units, mean ± s.e.m.) was calculated with ImageJ software (n= 4 animals per group, 8 sections per animal).

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Supplemental Figure 3

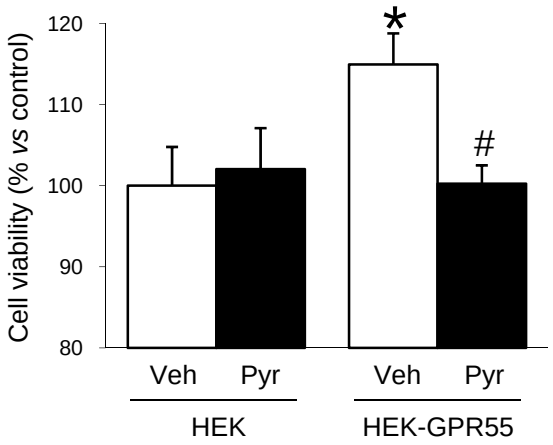

Figure S3. Inhibition of LPI synthesis prevents GPR55-induced increase in cell proliferation. HEK293 cells stably expressing 3xHA-GPR55 (HEK-GPR55) or the corresponding empty vector (HEK) were serum-starved overnight and incubated with the PLA_2 inhibitor pyrrophenone (Pyr, 1 μM, generously donated by Dr. Balsinde, Instituto de Biología y Genética Molecular, Valladolid, Spain) or vehicle (Veh) for 48 h. Cell viability was determined by the MTT test. Results are expressed as % *vs* the cell viability of vehicle-treated HEK293 cells, set at 100%. *, $p<0.05$ *vs* vehicle-treated HEK293 cells; #, $p<0.05$ *vs* vehicle-treated HEK293-GPR55 cells; n=3.

CHAPTER 2

In this second chapter, we will study in more detail the role of GPR55 in tumor generation and progression. To achieve this goal, we will use the well-established model of chemically-induced skin carcinogenesis in mice lacking GPR55 and their corresponding wild type littermates. We will study the effects of deleting GPR55 on malignant transformation, and we will also try to unravel the molecular bases of such effects. Finally, we will analyze the expression of GPR55 in different human squamous cell carcinomas and we will try to determine whether this expression correlates with markers with clinical relevance.

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ORIGINAL ARTICLE

The orphan receptor GPR55 drives skin carcinogenesis and is upregulated in human squamous cell carcinomas

E Pérez-Gómez[1], C Andradas[1], JM Flores[2], M Quintanilla[3], JM Paramio[4], M Guzmán[1,5] and C Sánchez[1]

G protein-coupled receptors (GPCRs) control crucial physiological processes and their dysfunction contributes to various human diseases, including cancer. The orphan GPCR GPR55 was identified and cloned more than a decade ago, but very little is known about its physio-pathological relevance. It has been recently shown that GPR55 controls the behavior of human cancer cell lines in culture and xenografts. However, the assessment of the actual role of this receptor in malignant transformation *in vivo* is hampered by the lack of studies on its functional impact in clinically-relevant models of cancer. Here we demonstrate that GPR55 drives mouse skin tumor development. Thus, GPR55-deficient mice were more resistant to DMBA/TPA-induced papilloma and carcinoma formation than their wild-type littermates. GPR55 exerted this pro-tumor effect primarily by conferring a proliferative advantage on cancer cells. In addition, GPR55 enhanced skin cancer cell anchorage-independent growth, invasiveness and tumorigenicity *in vivo*, suggesting that it promotes not only tumor development but also tumor aggressiveness. Finally, we observed that GPR55 is upregulated in human skin tumors and other human squamous cell carcinomas compared with the corresponding healthy tissues. Altogether, these findings reveal the pivotal importance of GPR55 in skin tumor development, and suggest that this receptor may be used as a new biomarker and therapeutic target in squamous cell carcinomas.

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Keywords: GPR55; G protein-coupled receptors; skin carcinogenesis; squamous cell carcinoma; cannabinoids

INTRODUCTION

G protein-coupled receptors (GPCRs) are the direct or indirect target of >50% of current therapeutic drugs. In the context of oncology, however, despite the increasing evidence showing a link between GPCR deregulation and cancer, just a few GPCRs are being exploited as targets of chemotherapeutic agents. Indirect lines of evidence suggest that the orphan GPCR GPR55 may have a role in cancer physiopathology. Thus, increased levels of the phospholipid lysophosphatidylinositol (LPI), a putative GPR55 endogenous ligand,[1] have been found in plasma and ascites from patients with ovarian cancer compared with healthy women or with women with non-cancerous pathologies.[2,3] In addition, epithelial cells[4] and fibroblasts[5] are able to generate mitogenic LPI upon ras-driven transformation. More recently, it has been shown that GPR55 modulates cancer cell migration[6] and proliferation[7,8] *in vitro*, and tumor growth in a xenograft-based model of glioblastoma.[7] However, the actual role of GPR55 in malignant transformation *in vivo* remains unknown. One of the best established paradigms for studying the mechanisms underlying this process is the mouse skin model of two-stage carcinogenesis.[9] Animals subjected to this experimental protocol evolve through different stages of cancer progression: first, in the 'initiation' phase, key genes are mutated in keratinocyte stem cells by topical exposure to the mutagenic agent 7,12-dimethylbenz[a]anthracene (DMBA).[9] After the initiation stage, the population of mutated cells expands as a result of the repeated topical application of a proliferation-inducing agent such as the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), UV radiation or wounding. During this 'promotion' stage, animals develop benign hyperplastic skin lesions termed papillomas.[9] With variable frequency these structures may progress to malignant invasive squamous cell carcinomas or the more aggressive spindle cell carcinomas. This 'progression' stage occurs independently of tumor promoters and is the consequence of the genetic alterations accumulated during the proliferation of initiated cells.[9] Here, by using this experimental protocol, as well as other *in vivo* and *in vitro* approaches, we demonstrate that GPR55 has an essential role in skin tumor development.

RESULTS

GPR55 is expressed in mouse epithelia

First, we analyzed the expression of GPR55 in different mouse-stratified epithelia. As shown in Supplementary Figure 1, the receptor was moderately expressed in mouse skin, specifically in the epidermis (Supplementary Figure 1A). A similar pattern of GPR55 expression was found in other mouse-stratified epithelia. Thus, a discreet GPR55 immunoreactivity was detected in the epithelial compartments of the oral cavity (Supplementary Figure 1B), esophagus (Supplementary Figure 1C) and stomach (Supplementary Figure 1D). Interestingly, GPR55-positive cells were mainly located in the most proliferative layers of those epithelia (Supplementary Figure 1). Controls of the specificity of GPR55 staining are provided in Supplementary Figure 2.

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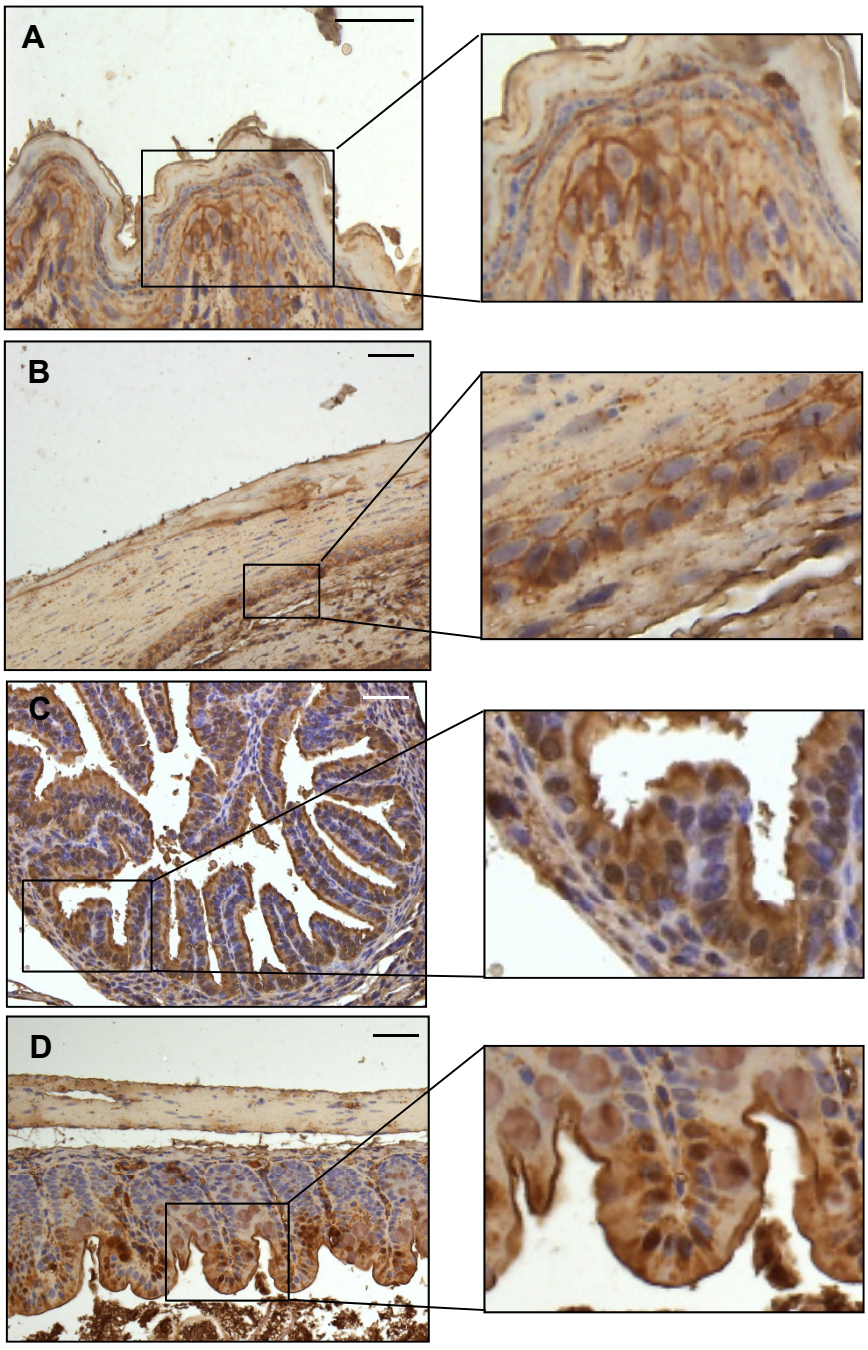
Supplementary Fig. S1



Supplementary Fig. S1 Expression of GPR55 in mouse stratified epithelia. Immunohistochemical analysis of GPR55 in mouse skin (A), oral cavity (B), esophagus (C) and stomach (D). Right images represent magnifications of the selected areas. GPR55 appears in brown and cell nuclei in blue. Scale bars, 200 μm.

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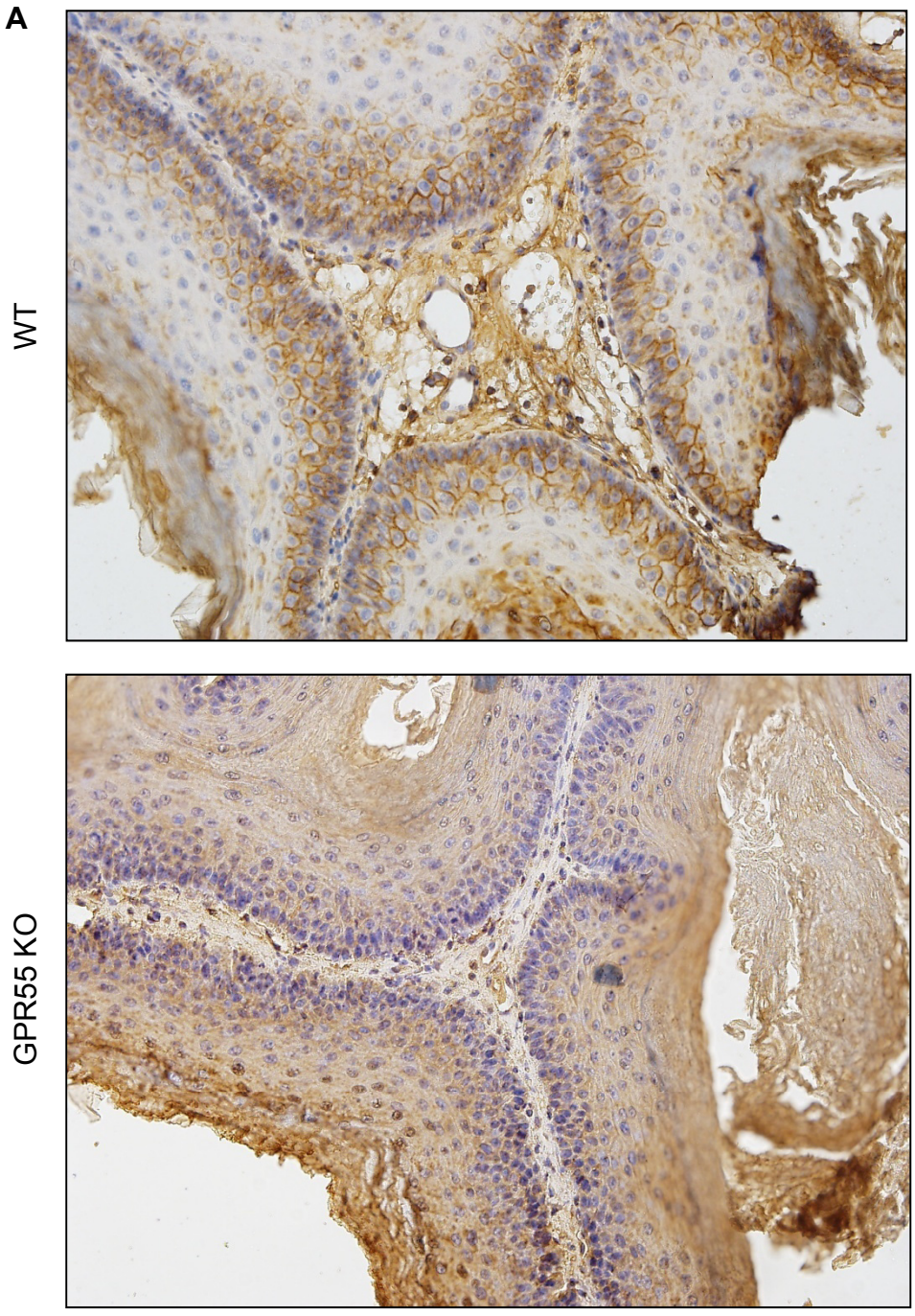
Supplementary Fig. S2



Immunohistochemical staining of GPR55 in papillomas generated in the skin of wild type (WT) and GPR55-deficient (GPR55 KO) mice. GPR55 appears in brown and cell nuclei in blue.

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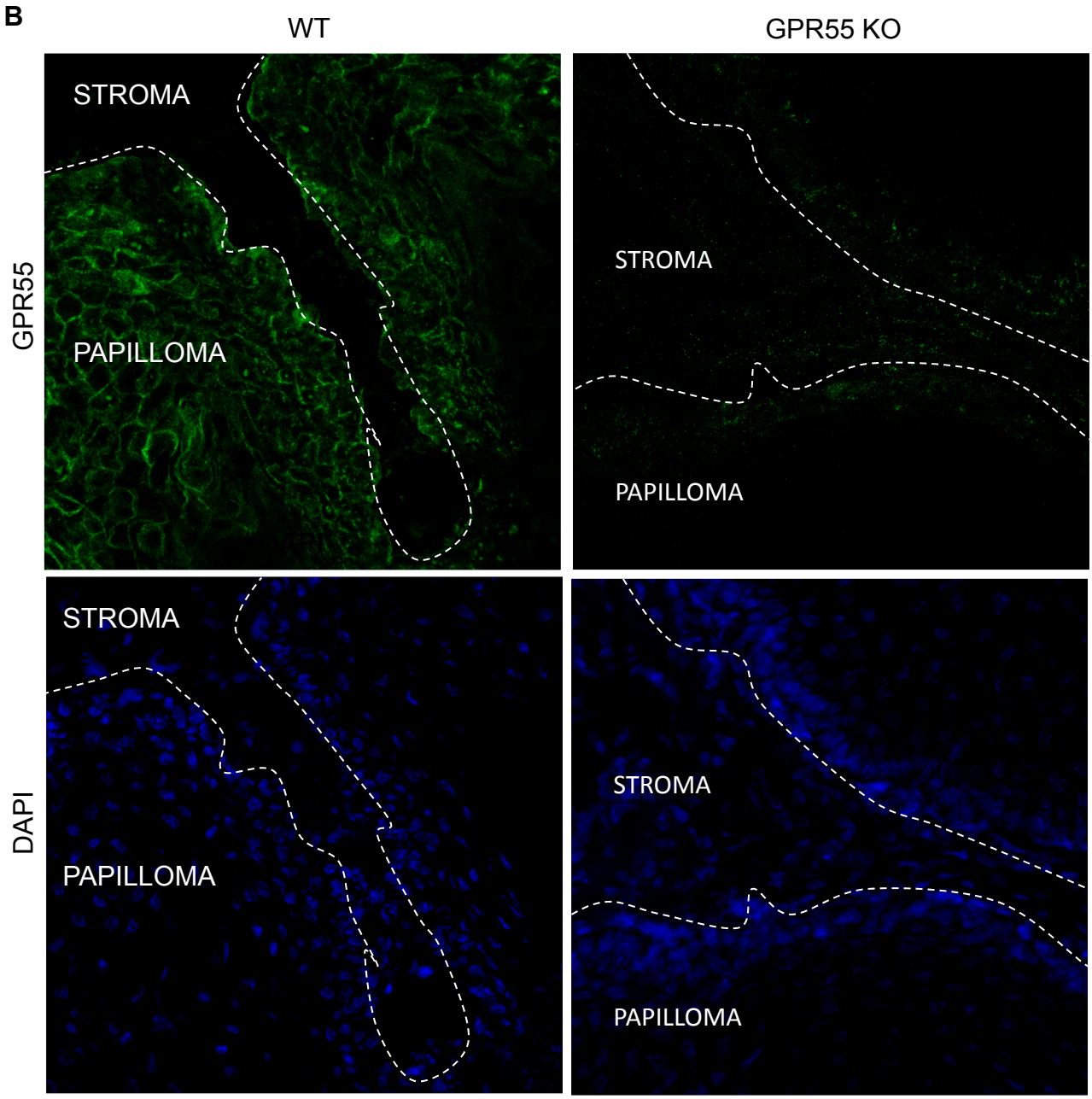
Supplementary Fig. S2



Immunofluorescence staining of GPR55 in papillomas generated in the skin of wild type (WT) and GPR55-deficient (GPR55 KO) mice. GPR55 appears in green and cell nuclei in blue.

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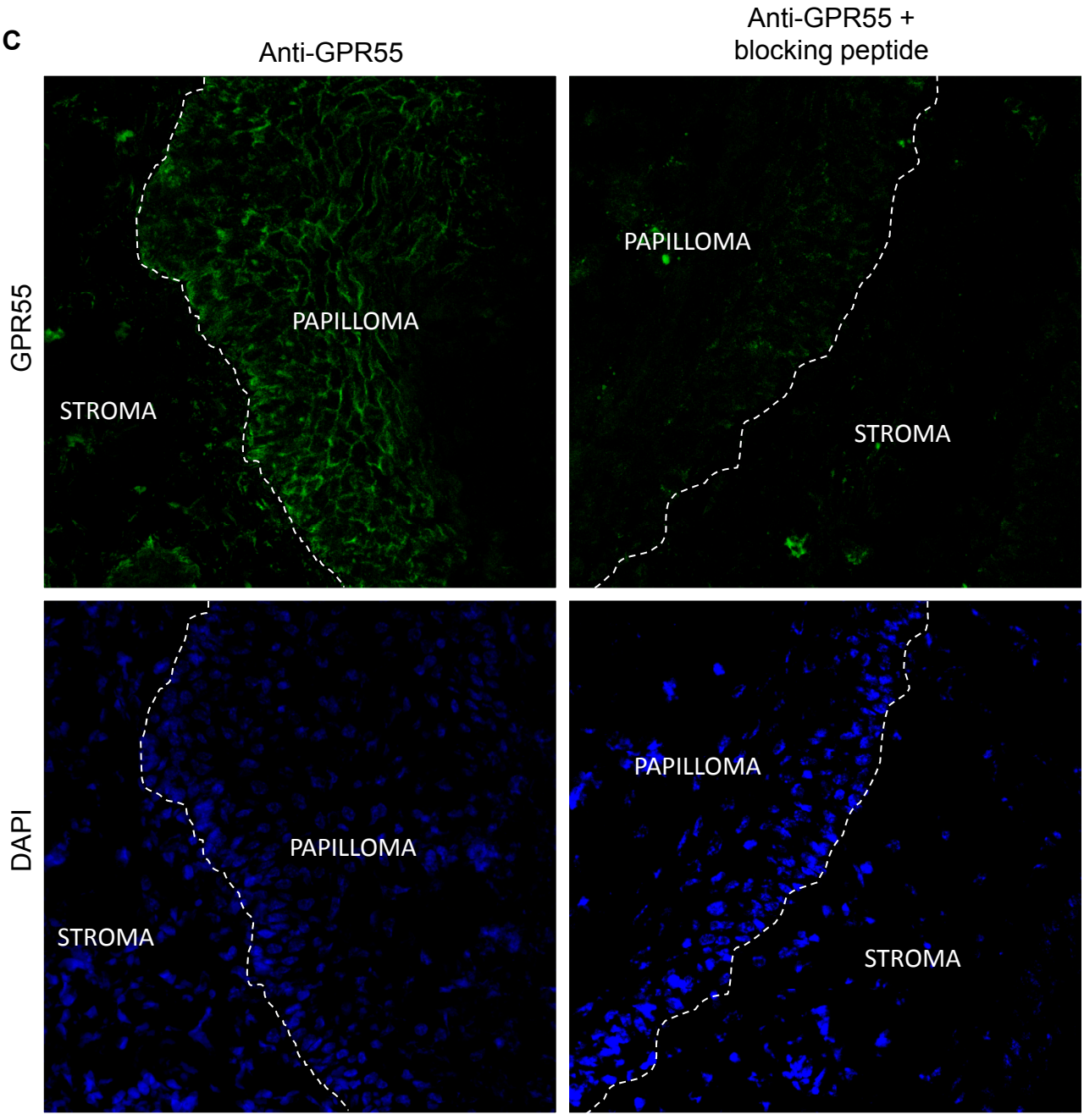
Supplementary Fig. S2



Immunofluorescence staining of GPR55 in a papilloma generated in the skin of a wild type mouse. Left panels, sections were incubated with anti-GPR55 antibody. Right panels, sections were incubated with the anti-GPR55 antibody preincubated with the corresponding blocking peptide. GPR55 appears in green and cell nuclei in blue.

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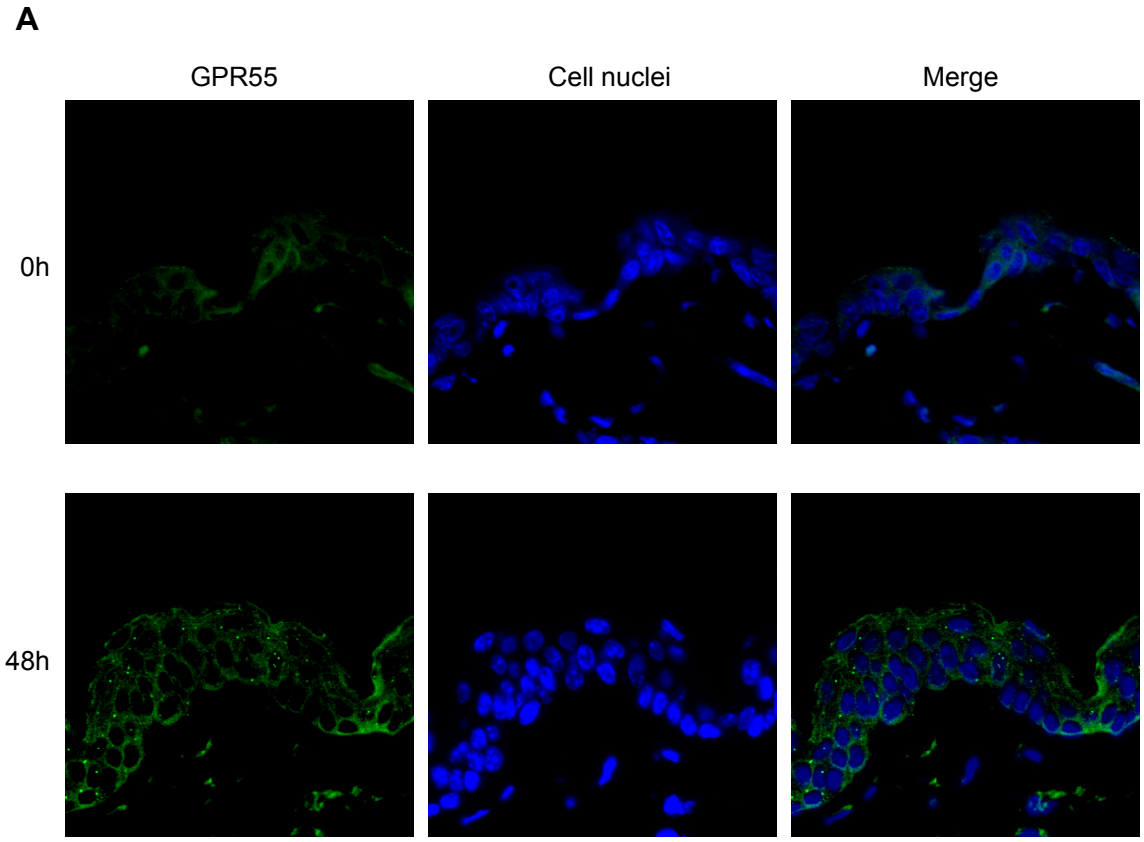
Supplementary Fig. S3



Supplementary Fig. S3 GPR55 is upregulated upon TPA-treatment. Wild type mice were treated topically with a single TPA application. GPR55 expression in the skin was analyzed by immunofluorescence at the indicated times.

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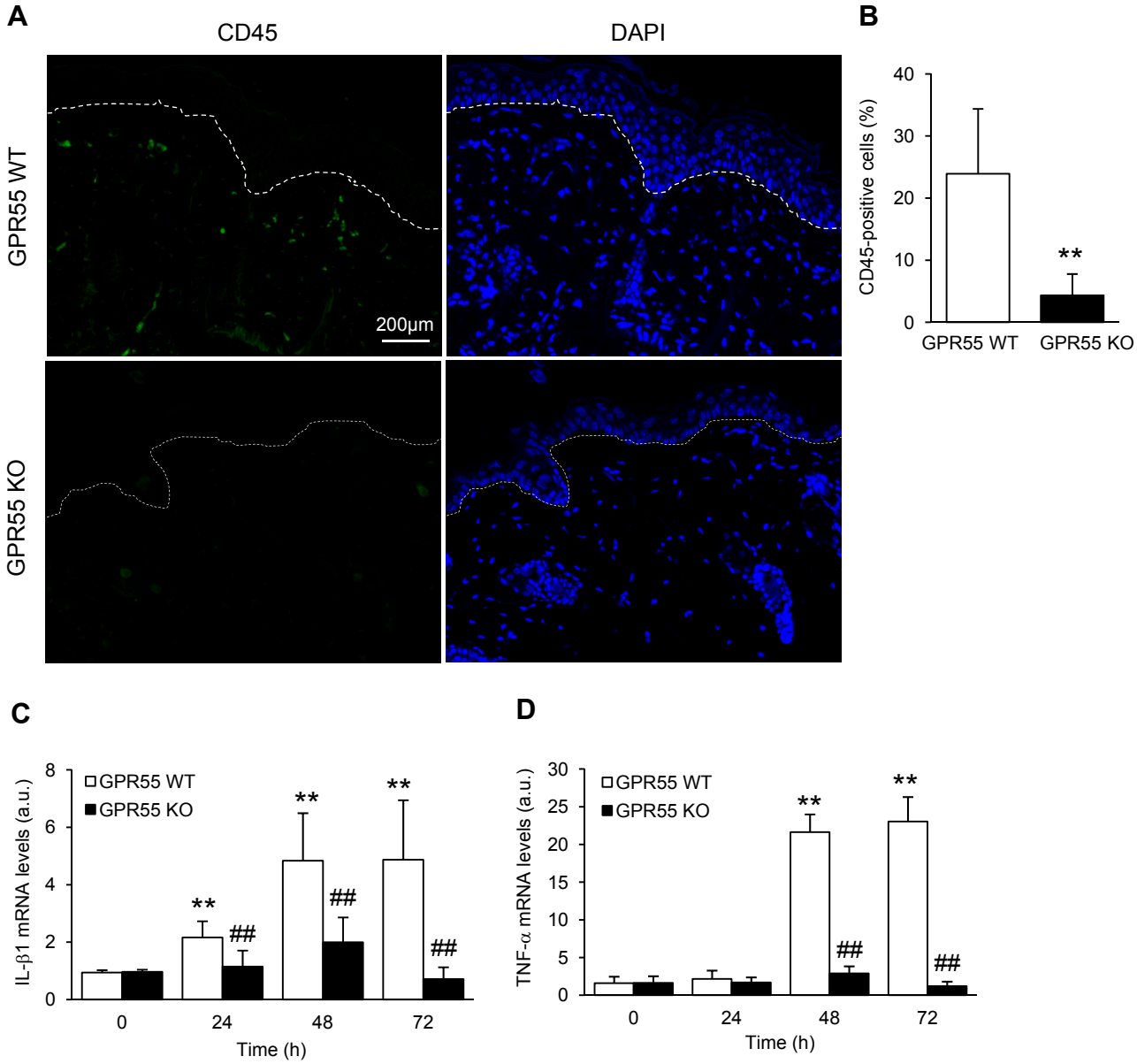
Supplementary Fig. S4



Supplementary Fig. S4 GPR55 mediates TPA-induced dermal inflammation. Wt and GPR55 KO mice received a single topical application of the tumor promoter TPA. Immunofluorescence analysis (A) and quantification (B) of CD45-positive (green) cells (differentiated hematopoietic cells except erythrocytes and platelets) 72 h after TPA application. Cell nuclei are in blue. Results are expressed as % *vs* total cells per field. C and D, Real-time quantitative PCR analysis of IL-β1 (C) and TNF-α (D) mRNA, in the skin of wt and KO mice at the indicated times after TPA application. Results are expressed in arbitrary units. **, p<0,01 *vs* t_0 (time of TPA application); ##, p<0,01 *vs* the corresponding group of wt mice.

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Supplementary Table S1. Primers used for reverse-transcriptase (RT) and real-time quantitative (RTQ) PCR

RT-PCR primers	**Sense**	**Antisense**
mGPR55	5′-GTGGTCTCCTTTCTCCCAGTG-3′	5′-TTAGCCCCTGGAGACCATGGTATCC-3′
mc-Fos	5′-AGCATGGGCTCTCCTGTCAA-3′	5′-GGGCTGCCAAAATAAACTCC-3′
GAPDH	5′-GGGAAGCTCACTGGCATGGCCTTCC-3′	5′-CATGTGGGCCATGAGGTCCACCAC-3′

RTQ-PCR primers	**Sense**	**Antisense**
hGPR55	5′-CTGCCTTGGTTCCACCATA-3′	5′-CCAGGATGCAGGTGAGTAAGA-3′
mGPR55	5′-TGGCCAGGCATCTTCAGT-3′	5′-CCAAGAGAAGTCCCCTTTCC-3′
mIL1β	5′-TGTAATGAAAGACGGCACACC-3′	5′-TCTTCTTTGGGTATTGCTTGG-3′
mTNFα	5′-TGCCTATGTCTCAGCCTCTTC-3′	5′-GAGGCCATTTGGGAACTTCT-3′
18S	5′-GCTCTAGAATTACCACAGTTATCCAA-3′	5′-AAATCAGTTATGGTTCCTTTGGTC-3′

CHAPTER 3

In this third chapter, we will investigate whether GPR55 participates in the metastatic process. Thus, we will analyze the effect of GPR55 on breast cancer cell migration and invasion *in vitro* and in tumor growth and lung colonization *in vivo*. For these studies we will use breast cancer cells with stably knocked-down GPR55 levels or transfected with the corresponding control. We will also study the downstream mechanisms responsible for the effects of GPR55 on the pro-metastatic-related features.

The following results are not publish yet. They are part of a future manuscript still in preparation.

The orphan receptor GPR55 confers pro-metastatic advantages on breast cancer cells *in vitro* and *in vivo*

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Abstract

Emerging evidence points to an important role of the orphan GPCR GPR55 on tumor generation and growth. Thus, we have previously demonstrated that GPR55 promotes cancer cell proliferation *in vitro* and *in vivo*. In these work we aimed at studying whether GPR55 participates in the control of the metastatic process. We demonstrate that GPR55 drives breast cancer cell migration and invasion via G_q heteromeric G proteins *in vitro*. This effect was accompanied by the activation of MMPs and the upregulation metastatis-inducing genes. Furthermore, GPR55 promotes breast cancer tumor growth and lung colonization *in vivo*. Together, our data support the involvement of GPR55 on the final and most lethal stage of cancer progression, and suggest that pharmacological blockade of this receptor could be a new strategy to manage metastatic cancer.

Introduction

Metastasis is the last step in tumor progression. While non-invasive tumors are usually benign, invasive tumors are the cause of 90% of cancer-associated deaths (Gupta and Massagué, 2006). Metastatic cancer cells present the ability to leave the primary tumor and colonize distal niches. To do so, cancer cells acquire several capabilities that allow them to detach from their neighboring cells and the extracellular matrix, to migrate to and enter the circulatory system, to express survival signals that allow them to travel across the blood stream to distant tissues and to colonize them generating new tumoral masses (Hanahan and Weinberg, 2011). The identification of the genes and mechanisms governing all these steps, and therefore the metastatic process, is essential for understanding cancer progression biology and for the development of novel therapeutic tools to prevent/treat metastasis.

The orphan receptor GPR55 has been recently involved in tumor generation and growth (Andradas et al., 2011; Pérez-Gómez et al., 2012; Piñeiro et al., 2011). Thus, GPR55 promotes cancer cell proliferation, both *in vitro* and *in vivo*, and confers oncogenic advantage to these tumoral cells. In line with this notion, GPR55 appears correlated to the aggressiveness of breast, brain and pancreatic human cancers (Andradas et al., 2011).

Indirect evidence suggests that GPR55 may also be involved in tumor metastasis. First, GPR55 couples to $G\alpha_{12/13}$ (Balenga et al., 2011; Brown et al., 2011; Henstridge et al., 2009; Huang et al., 2011; Lauckner et al., 2008; Obara et al., 2011; Oka et al., 2010; Ryberg et al., 2007) and $G\alpha_q$ (Lauckner et al., 2008; Obara et al., 2011; Waldeck-Weiermair et al., 2008) heterotrimeric G proteins that have been related to tumor progression in general and cancer cell migration and invasion in particular (Dorsam and Gutkind, 2007; Kelly et al., 2007; Kim et al., 2011; Worzfeld et al., 2008). In addition, GPR55 activates small Rho GTPases (Balenga et al., 2011; Henstridge et al., 2009; Lauckner et al., 2008; Obara et al., 2011; Oka et al., 2009; Ryberg et al., 2007; Whyte et al., 2009), proteins that regulate actin cytoskeleton organization, cell-cell adhesion and cell polarity among others (Kelly et al., 2007), cell functions that are intimately related to the capability of cells to invade and migrate and, therefore, to metastasize (Karlsson et al., 2009). More recently, GPR55 has been shown to promote anchorage-independent growth. Thus, down-regulation of GPR55 in PDV skin carcinoma cells (Pérez-Gómez et al., 2012) and PC-3 prostate cancer cells (Piñeiro et al., 2011), inhibited cell growth in soft agar. Moreover, overexpression of GPR55 in the poorly metastatic breast cancer cell line MCF-7 confers a migratory phenotype to these cells, which is enhanced by LPI treatment and prevented by selective down-regulation of GPR55 (Ford et al., 2010).

In this context, we aimed at studying in depth the involvement of GPR55 in cancer cell invasion and migration. Our results show that GPR55 enhances the invasion and migration properties of the human breast adenocarcinoma cell line MDA-MB-231 *in vitro* via $G\alpha_q$ proteins, and promotes tumor growth and lung colonization *in vivo*.

Materials and methods

Cell culture

MDA-MB-231 human adenocarcinoma cell line was supplied by ATCC-LGC. These parental cell line and those derived from it were maintained in DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and incubated at 37ºC and 5% CO_2.

Plasmid transfection and stable cell line generation

To generate the stably GPR55 knocked-down MDA-MB-231 cell line, double-stranded oligonucleotides encoding short hairpin RNA (shRNA) against human GPR55 were inserted into the psi-U6-EGFP vector (shGPR55) (GeneCopoeia, Rockville, MD). Cells were transfected with shGPR55 or the corresponding control (psi-U6-EGFP-unrelated sequence vector) with Lipofectamine 2000 according to manufacturer's instructions (Invitrogen, Barcelona, Spain). Transfected cells were selected with gradually increasing concentrations of puromycin (up to 20 µg/mL) (Sigma, St. Louis, MO), and the most intense GFP pool was isolated using a FACS Vantage sorter (Becton Dickinson, Madrid, Spain).

For transient transfection, the following plasmids were introduced into the cells using Lipofectamine 2000 as transfection reagent: 3xHA-human recombinant GPR55 plasmid (Henstridge et al., 2009) or the corresponding empty vector (pcDNA3); pCEFL vectors containing GFP, GFP fused to the RGS domain of PDZ-RhoGFP (GFP-RGS) or GFP fused to the RGS domain of GRK2 (GFP-GRK2), all of them kindly donated by Dr. Gutkind (NIH, Bethesda, MD). 24 h after transfec-

tion, cells were serum starved for invasion assays.

Lentiviral infections

To generate MDA-MB-231-luciferase cell lines, cells were infected with lentiviral particles containing luciferase (kindly donated by Dr. Gutkind). shC and shGPR55 MDA-MB-231 cells at ~50% confluence were incubated with viral supernatants for 24 h at 37ºC in the presence of 8 μg/mL polybrene (hezadimethrine, Sigma). Cells were washed with PBS and incubated in 10% FBS-media until use.

Real-time quantitative PCR

RNA from cell cultures was isolated with Trizol Reagent (Sigma) including a DNase digestion step, with the DNase I recombinant (Roche, Basel, Switzerland). cDNA was subsequently obtained with Transcriptor Reverse Transcriptase (Roche). Real-time quantitative PCR assays were performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library (Roche). The primers used for human GPR55 were: sense, 5'-CATGTGTTTCTCCAACGTCAA-3' and anti-sense, 5'-TGCGGAATTCTTTGATGACA-3'; for human ANGPTL4: sense, 5'-GGAACAGCTCCTGGCAATC-3' and anti-sense, 5'-GTTGACCCGGCTCACAAT-3'; for human MMP1: sense, 5'-GTCCTTGGGGTATCCGTGTA-3' and anti-sense, 5'-ACGAATTTGCCGACAGAGAT-3'; and for CXCL1 sense, 5'-CTTCAGGAACAGCCACCAGT-3' and anti-sense, 5'-CATCGAAAAGATGCTGAACAGT-3'. Amplifications were run in a 7900 HT-Fast Real Time PCR System (Applied Biosystems, Carlsbard, CA). Each value was adjusted by using 18S RNA levels as reference, amplified with multispecies primers sense, 5'-GCTCTAGAATTACCACAGTTATCCAA-3' and anti-sense, 5'-AAATCAGTTATGGTTCCTTTGGTC-3'.

Western blot analysis

Tumors and cell lines were lysed on a buffer containing 50 nM tris HCl, 1 mM phenylmethylsulfonyl fluoride, 50mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% Triton X-100, 1 μg/mL leupeptin, 1 mM EDTA, 1 mM EGTA and 10 mM sodium β-glicerophophate at pH 7.5 supplemented with a protease inhibitor cocktail (Roche), PMSF 200 μM, microcystine 200 μM and β-mercaptoethanol 200 μM. Lysates were subjected to PAGE-SDS, and proteins transferred onto polyvinylidene fluoride membranes. Blots were incubated with the following antibodies: anti-HA (Roche), anti-GFP (Roche) and anti-β-actin (Sigma) used as leading control. Luminograms were obtained with the Amersham Enhanced Chemiluminescence Detection Kit (GE Healthcare).

Migration assays

Migration assays were performed in a 48-well Boyden chambers with polyvinyl pyrrolidone free polycarbonate membranes of 8 μm of pore size (NeuroProbe, Gaithersburg, MD) previously coated with fibronectin (10 μg/mL) for 7-8 h at 4ºC. Chemoattractants [LPI (Sigma) or EGF (Chemicon, Temecula, CA)] were added to the lower chamber in serum-free media. Cells were serum starved over night and added at a density of 15000 cells/well into the upper chamber in serum-free media. After 8 h of incubation at 37ºC, cells were fixed in methanol. Cells on the upper surface of the membranes were wiped with a cotton swab and those that underwent (i.e. in the lower surface) were stained with hematoxilyn and counted.

Invasion assays

Invasion assays were performed in BD BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences, Bedford, MA). Matrigel inserts were rehydrated with serum-free media. Chemoattractants [FBS or LPI (0.5 μM)] were added into the wells, and serum starved cells (100000/well) were added into the inserts. Plates were incubated for 24 h at 37ºC and 5% CO_2. Cells were then fixed in 4% formaldehyde, washed with PBS and stained with DAPI (100 ng/mL) (Roche). Images from inserts were captured in z-stack for 3D reconstruction with confocal microscope Leica TCS SP5. Images were processed with Imaris x64 7.3.1 software.

MMP activity assay

MMP2 (Gelatinase A) and MMP9 (Gelatinase B) activities were determined by gelatin zymography. Media collected from cell cultures were run in PAGE-SDS gels in the presence of 0.1% gelatin. Gels were then washed with a 2.5% Triton X-100 containing buffer to renaturalize and activate the gelatinases, and incubated overnight at 37°C in 50 mM Tris, 150 mM NaCl, 10 mM CaCl2, 0.1% Triton X100 pH 7.5. Gels were then stained with Coomasie Blue and washed with 8% acetic acid-20% methanol. Digested bands were quantified by densitometric analysis with Quantity One software (Bio-Rad).

Animal experiments

All procedures involving animals were performed with the approval of the Complutense University Animal Experimentation Committee in compliance with the European official regulations. For orthothopic breast cancer assays, $2x10^6$ cells in 50 μL of PBS were injected into the 4th right mammary-fat-pad of anesthesized 6-week old SCID/NOD female mice. Animals were randomly assigned in two groups: 10 mice were injected with the shC MDA-MB-231 cell line and 15 mice with the shGPR55 MDA-MB-231 cell line. Tumors were measured once a week with external caliper and volume was calculated as $(4\pi/3) \times (width/2)^2 \times (length/2)$. Mice were sacrificed 60 days after cell injection and lungs, intestine, pancreas, spleen, brain and tumors were collected. All tissues were fixed in 4% formalin and tumors were divided into three portions: 1) fixed in 4% formalin, 2) snap-frozen for protein extraction and 3) snap-frozen for RNA extraction. Samples for protein and RNA analysis were store at -80ºC until use.

For lung metastases analysis, $5x10^5$ luciferase-expressing cells, resuspended in 100 μL of PBS, were injected into the lateral tail vein of 6-week old SCID/NOD mice (4 per group). Thirty days after cell injection, animals were analyzed by bioluminescence imaging. Mice received an intraperitoneal injection of D-luciferin (Gold Biotechnology, St Louis, MO), and 15 min later luciferase signal was captured by an IVIS 2000 system (Xenogen Corp, Alameda, CA). Imaging data were processed with Living Image software (Xenogen Corp) and analyzed using the average radiance ($p/s/cm^2/sr$) of the regions of interest (ROI). Animals were sacrificed and lungs were snap-frozen and store at -80ºC until use.

Statistical analysis

Statistical analysisANOVA with a post-hoc analysis by the Student–Newman–Keuls' test was routinely used. Unless otherwise stated, data are expressed as mean±s.e.m.

Results and discussion

GPR55 promotes breast cancer cell migration

To analyze the role of GPR55 on the metastatic potential of cancer cells, we first studied whether this receptor modulates the migration properties of the highly metastatic breast cancer cell line MDA-MB-231. We performed *in vitro* migration assays in Boyden chambers using LPI, a potential GPR55 endogenous ligand, as chemoattractant. MDA-MB-231 cells, which express elevated levels of GPR55 (Andradas et al., 2011; Ford et al., 2010), migrated towards LPI in a dose-dependent manner, following a Gaussian pattern, being 0.5 μM LPI the most efficient chemoattractant concentration (figure 1A). Stably knock-down of GPR55 by selective shRNA (figure 1B) significantly impaired migration towards LPI (figure 1A). GPR55 knock-down did not affect the migration response towards epidermal growth factor (EGF), a ligand that binds to the tyrosine kinase receptor EGFR (figure 1A). These results indicate that GPR55 promotes the migration of breast cancer cells.

GPR55 enhances the invasiveness of breast cancer cells

We next analyzed whether GPR55 regulates the invasive capacity of breast cancer cells, another feature that is intimately related to their metastatic potential. In this case, we carried out invasion assays in matrigel Boyden chambers. MDA-MB-231 cells showed an invasive response towards both FBS and LPI (figure 2A and 2B). This effect was significantly reduced when GPR55 was stably knocked-down (figure 2A and 2B). Moreover, when GPR55 expression was reconstituted (figure 2C) tumor cells increased their invasiveness towards FBS and LPI (figure 2D and 2E).These data support that GPR55 promotes the invasion of breast cancer cells.

GPR55 signals breast cancer cell invasion through G_q proteins

It has been described that GPR55 couples to $G_{12/13}$ and G_q (Henstridge et al., 2011; Ross, 2009). Both classes of heterotrimeric G proteins

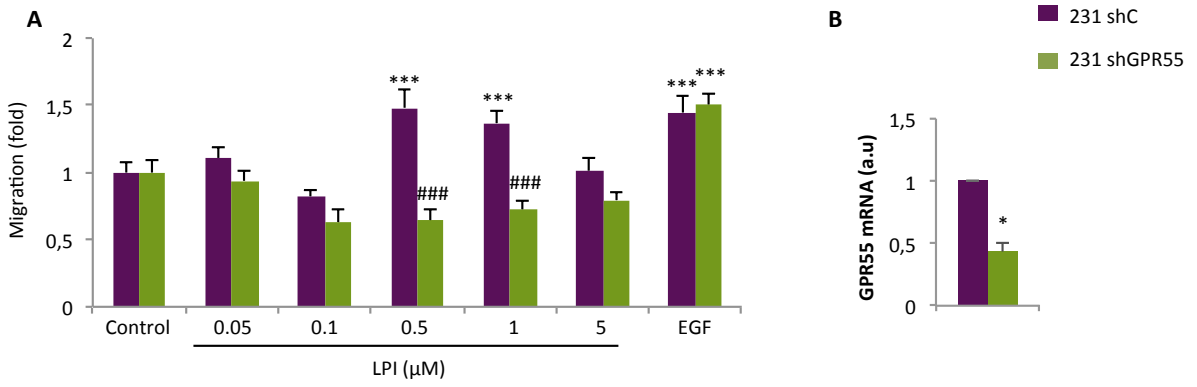

Figure 1. GPR55 promotes breast cancer cell migration. *A, Fraction of MDA-MB-231 cells that underwent migration towards increasing concentrations of LPI and 50nM of EGF or their corresponding vehicle (control). Green bars correspond to MDA-MB-231 cells with stably knocked-down GPR55 expression (231 shGPR55), while purple bars represent shControl transfected cells (231 shC). B, Relative GPR55 mRNA levels, as determined by real-time quantitative PCR, in 231 shC and 231 shGPR55 cells. *p<0.05; ***p<0.001 vs vehicle treated cells and ###p<0.001 vs the corresponding LPI treatment in 231 shC cells.*

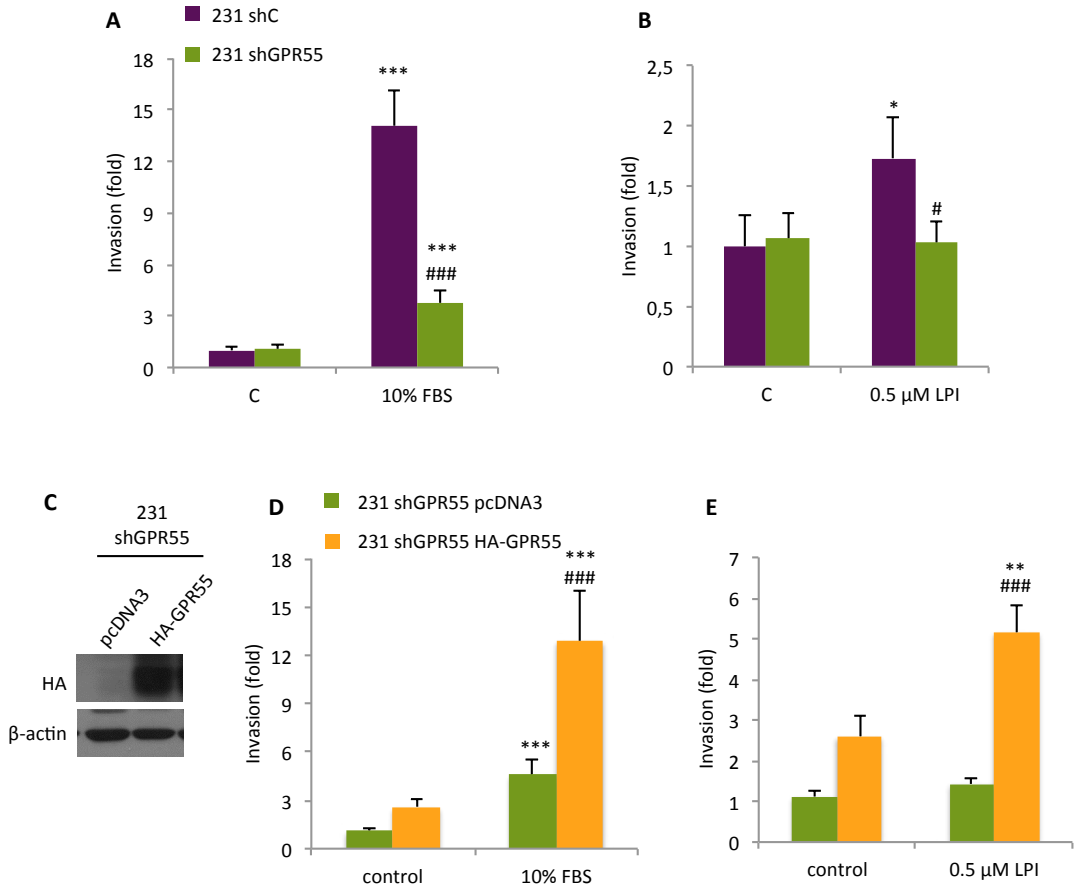

Figure 2. GPR55 enhances breast cancer cell invasiveness. *Fraction of cells that underwent invasion in matrigel Boyden chamber towards FBS (A) or LPI (B). Green bars represent MDA-MB-231 cells with stably knocked-down expression of GPR55 and purple bars correspond to cells stably transfected with a control shRNA. C, analysis of GPR55 protein expression (by western blot with anti HA antibody) in shGPR55 MDA-MB-231 cells transfected with a HA-GPR55 plasmid (231 shGPR55 HA-GPR55) or the corresponding empty vector (231 shGPR55 pcDNA3). D and E, invasion of 231 shGPR55 pcDNA3 and HA-GPR55 cells towards FBS (D) or LPI (E). *p<0.05; **p<0.01; ***p<0.001 vs vehicle treated cells and #p<0.05; ###p<0.001 vs the corresponding invasion towards FBS or LPI of 231 shC cells or 231 shGPR55 pcDNA3 cells.*

have been implicated in tumor progression and metastasis (Dorsam and Gutkind, 2007; Kelly et al., 2007). Consequently, we next examined whether the GPR55-induced enhancement of cancer cell invasion was mediated by coupling of the receptor to either $G_{12/13}$ or G_q. We used two chimeric constructions that behave as dominant negative mutants for $G_{12/13}$ and G_q. These constructs consist of the green fluorescent protein (GFP) fused either to the RGS domain of PDZ-RhoGEF, that binds to and impairs activation of $G_{12/13}$, or to the RGS domain of GRK2 that binds to G_q blocking its signaling pathway. Cells were transfected with either construct (figure 3A) and invasion assays were performed. Blocking of $G_{12/13}$ signaling with the GFP-RGS chimera did not affect the enhanced invasion towards FBS or LPI produced by GPR55 overexpression (figure 3B and 3C). In contrast, impairement of G_q signaling with the GFP-GRK2 construction blocked the increase in the invasive potential of cancer cells induced by GPR55 overexpression (figure 3D and 3E). These data indicates that GPR55 enhances breast cancer cell invasion via G_q signaling.

GPR55 downregulation reduces MMP2 and MMP9 activity

Extracellular matrix remodeling is crucial during cancer progression. This process is carried

out by different proteinases, being matrix metalloproteinases (MMPs) one of the most representative members. MMPs present proteolytic activity that degrades physical barriers during cancer progression, at the local site where tumors are primarily originated during the intravasation and extravasation processes, and at the distant organs where metastases are generated (Gialeli et al., 2010; Hua et al., 2011). Since tumor invasion and metastasis are often associated with enhanced activity of MMP2 and MMP9, we examined whether GPR55 modulates the activity of these

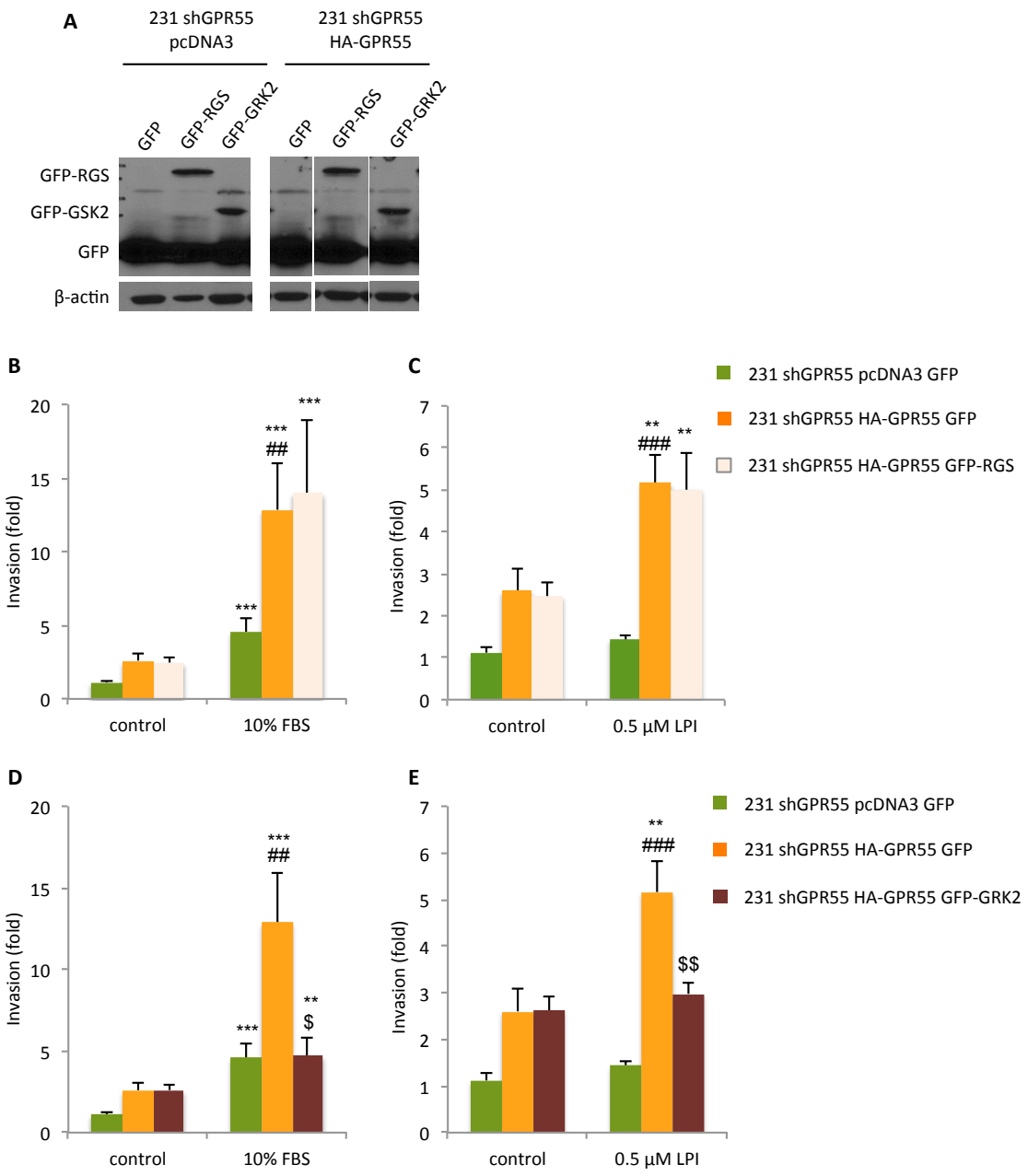

Figure 3. GPR55 signals breast cancer cell invasion through G_q. *MDA-MB-231 cells with stably knocked-down levels of GPR55 were transfected either with a HA-GPR55 plasmid (231 shGPR55 HA-GPR55) or with the corresponding empty vector (231 shGPR55 pcDNA3), and one of the following constructs: GFP-RGS domain of PDZ-RhoGEF (GFP-RGS), GFP-RGS domain of GRK2 (GFP-GRK2) or the corresponding vector (GFP). A, expression of the fusion proteins was confirmed by Western Blot analysis with an anti-GFP antibody. β-actin was used as loading control. in MDA-MB-231 cells. B-E, cell invasion was determined as in figure 2 and is represented as faction of cells that underwent invasion towards FBS (B) and (D) or LPI (C) and (E). **p<0.01; ***p<0.001 vs the corresponding vehicle treated cells; #p<0.05;##p<0.01; ###p<0.001 vs invasion of 231 shGPR55 pcDNA3 GFP cells towards FBS or LPI and $p<0.05; $$p<0.01 vs invasion of 231 shGPR55 HA-GPR55 GFP.*

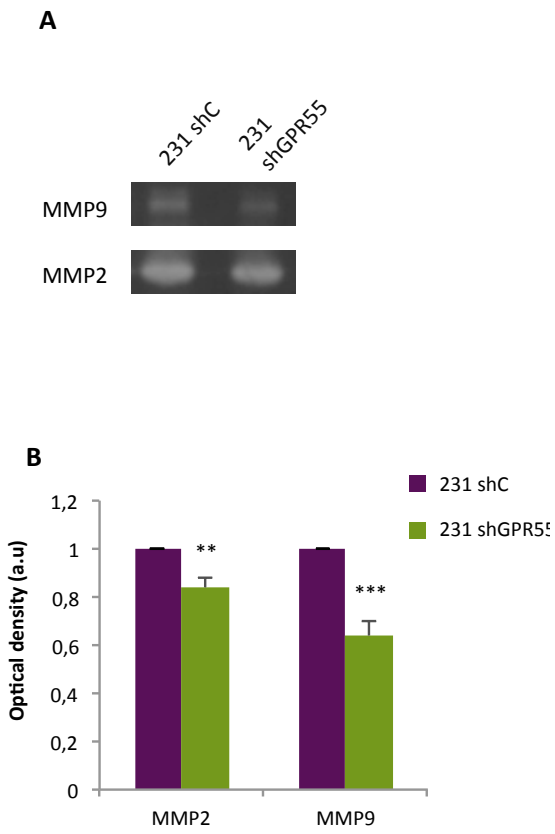

Figure 4. GPR55 downregulation decreases MMP2 and MMP9 activity. *A, Representative gelatin zymogram of media collected from MDA-MB-231 cells with stably GPR55 knocked-down levels (231 shGPR55) or stably transfected with a control shRNA (231 shC) 48 h after cell seeding. B, densitometric analysis of three independent experiments. Results are expressed in arbitrary units. **p<0.01; ***p<0.001 vs 231 shC.*

tow MMPs. By using gelatin zymogram assays with media from MDA-MB-231 cells, we observed that MMP2 and MMP9 activities were significantly reduced when GPR55 expression was stably knocked-down (figure 4). These data suggest that the invasive phenotype associated to elevated levels of GPR55 may be due, at least in part, to enhanced MMP2 and MMP9 activities.

GPR55 promotes tumor growth and invasion in vivo

We finally analyzed whether GPR55 modulates breast cancer progression *in vivo*. First, orthotopic breast tumors were generated by injection of MDA-MB-231-derived cell lines into the mammary fat pad of NOD/SCID mice. As show in figure 5, stable downregulation of GPR55 significantly reduced tumor growth (figure 5A). Next, we analyzed the involvement of GPR55 in two steps of the metastatic process closely connected to the invasive potential of cancer cells: the extravasation and colonization of distant tissues. MDA-MB-231 derived luciferase-expressing cell lines were injected in the tail vein of immunedeficient mice and lung metastases were evaluated 30 days after cell injection. Although, due to the reduced number of animals per group, no significant differences were observed, animals injected with 231 shC cells tended to present higher lung metastasis than those injected with GPR55 knocked down cells (figure 5B and 5C).

Different studies have determined that the induction of specific genes determines the capacity of breast cancer cells to generate metastasis specifically in the lungs. Some of them are matrix metalloproteinase 1 (MMP1), chemokine CXCL1 and angiopoetin-like 4 (ANGPTL4) (Minn et al., 2005; Padua et al., 2008). We analyzed by real-time quantitative PCR, the expression of these genes in the metastatic lungs generated in our tail vein injection-experiments. The mRNA expression of these three markers was significantly reduced in metastases generated from GPR55 stably knocked-down cells (figure 5D).

Together, these results indicate that GPR55 promotes tumor progression *in vivo*, by conferring a more metastatic phenotype to breast cancer cells.

Metastasis is the principal cause of cancer-associated deaths, and is produced by a lethal combination of intrinsic properties of the tumor

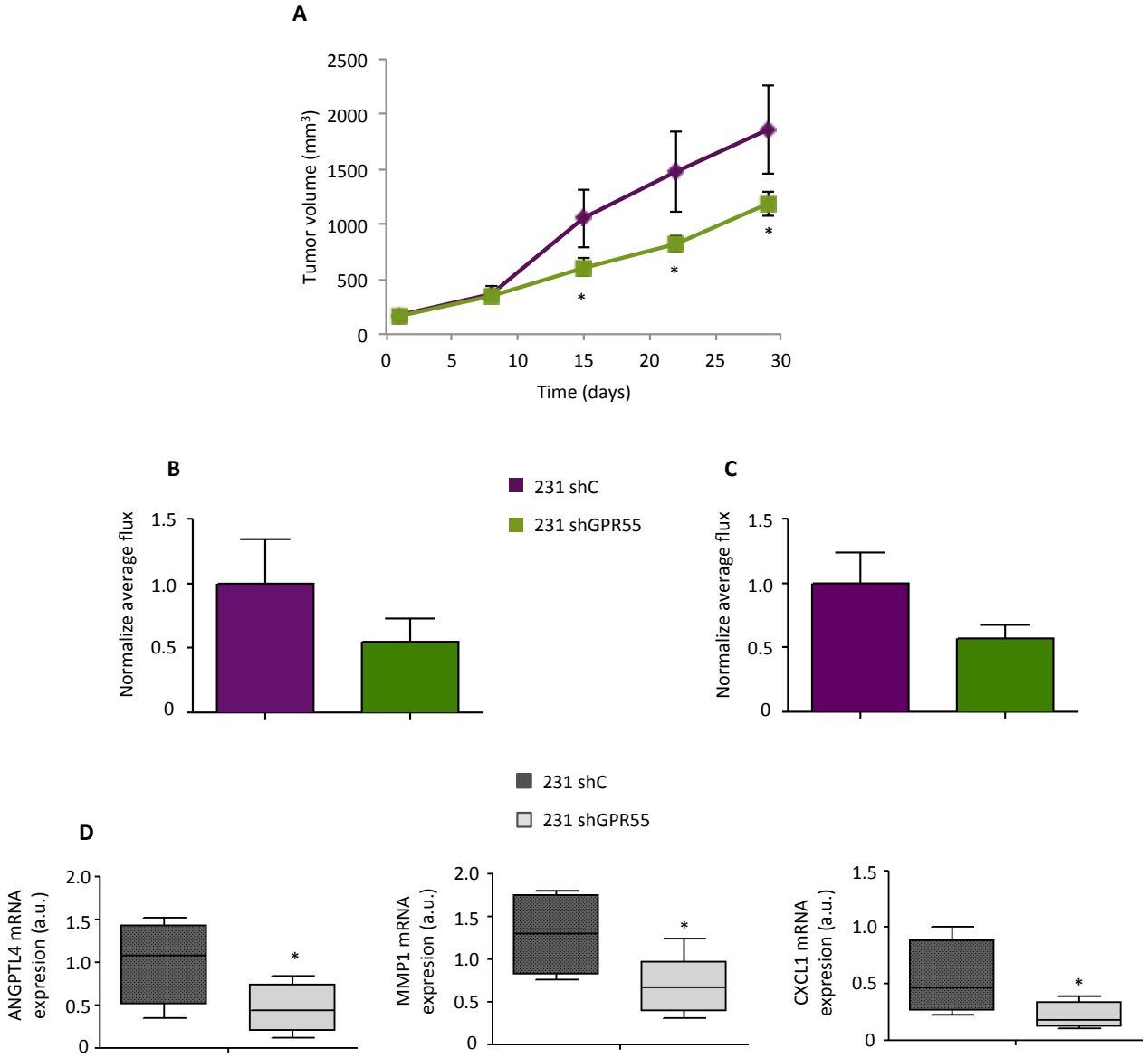

Figure 5. GPR55 promotes breast tumor growth and invasiveness in vivo. *A, Volume of tumors generated in the mammary fat pad of immune deficient mice by injection of MDA-MB-231 cells with stably downregulated levels of GPR55 (231 shGPR55) or stably transfected with a control shRNA (231 shC). B and C, evaluation of lung metastases (generated by tail vein injection of MDA-MB-231-derived luficerase-expressing cells) as determine by luciferase activity measurements in the whole animal (B) or specifically in the lungs (C). Results are expressed as normalized values of average flux vs 231 shC. D, angiopoetin-like 4 (ANGPPTL4), matrix metalloproteinase 1 (MMP1) and CXCL1 mRNA levels, as determine by real-time quantitative PCR, in the metastatic lungs. Results are expressed in arbitrary units. *p<0.01 vs 231 shC tumors.*

cells and signals coming from the tumor microenvironment and from different organs of the body (Ben-Baruch, 2007; Nguyen and Massagué, 2007). Here we show that the LPI/GPR55 system contributes to breast cancer progression. On one hand, GPR55 expression confers pro-metastatic advantages on cancer cell directly by enhancing their migration and invasion capabilities. In addition, we show that LPi behaves as a chemoattractant on GPR55 expressing cells, suggesting that it may contribute to the communication between tumor cells and the environment. This hypothesis is supported by the fact that increased circulating levels of LPI from ovarian cancer patients are associated with late-stage cancers or recurrence (Sutphen et al., 2004; Xiao et al., 2000). Similar associations have been found for other lysophospholipids. For example, modulation of systemic S1P, either by inhibiting its synthesis or by blocking S1P with a specific monoclonal antibody, re-

duced prostate cancer growth and lung metastasis generation (Ponnusamy et al., 2012).

Our results show that GPR55-induced actions on breast cancer cell invasion are mediated by G_q heterotrimeric G proteins. However, the specific downstream effectors regulating the invasive and migratory phenotype are still unknown. Elucidating these effectors and the associated signaling pathways may facilitate the pharmacological modulation of GPR55-driven cancer progression.

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Recent reports have described that GPR55 can be activated by different cannabinoid compounds. In addition, it is well established that cannabinoids control cell proliferation. Thus, in this last chapter we will study whether GPR55 mediates the effects of THC on cancer cell proliferation. First, we will analyze the consequence of modulating GPR55 expression on THC action. Then, we will try to unravel how THC produces such effects via GPR55. In particular, we will study whether THC promotes a release of intracellular calcium via GPR55 and whether THC action is produced by functional interaction of GPR55 with classical cannabinoid receptors.

The following results are not publish yet. They are part of a future manuscript still in preparation.

GPR55 participates in the effect of THC on cancer cell proliferation via CB_1/GPR55 functional interaction

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Abstract

GPR55 is an orphan G protein-coupled receptor that has been proposed to be engaged and activated by lysophospholipids (specifically by L-α-lysophosphatidylinositol) and cannabinoids (the active components of marijuana and their derivatives). It is well established that cannabinoids control cancer cell proliferation. Thus, these compounds generally induce a biphasic effect: while low concentrations of cannabinoids induce cancer cell proliferation, high concentrations exert the widely reported anti-proliferative action. In this work we aimed at studing the potential participation of GPR55 in the effect of THC on cancer cell proliferation. We show that GPR55 mediates the induction of cell proliferation by low concentrations of THC in different cancer cell types. Moreover, we describe a functional interaction between GPR55 and the classical cannabinoid receptor CB_1. Our data suggest that different GPR55/CB_1 interaction statuses may provide a mechanistic expanation for the biphasic effects of THC on cell proliferation.

Introduction

During the last decades, different findings have made possible the characterization of a novel cellular communication network named as the "endocannabinoid system" (ECS). This system consists of different membrane receptors, mainly CB_1 and CB_2, their specific endogenous ligands, known as endocannabinoids, and the enzymes involved in their synthesis and degradation (Lichtman et al., 2010). The ECS controls a wide variety of biological functions such as pain, motor coordination or appetite among other functions.

A family of compounds synthesized by the plant *Cannabis sativa* (marijuana) has the ability to activate this system. The main representative of this family, due to its abundance in the plant and its psychotropic potency is Δ^9-tetrahydrocannabinol (THC) (Mechoulam and Gaoni, 1967). THC, by binding to the same cannabinoid receptors than endocannabinoids, exert a variety of effects such as analgesia, appetite stimulation or neuroprotection, just to name a few. In the last 15 years, evidence has accumulated demonstrating that endogenously-produced, plant-derived and synthetic cannabinoids exert antitumoral responses. Specifically, cannabinoids present anti-proliferative, pro-apoptotic, anti-angiogenic and anti-invasive properties both *in vitro* and in animal models of cancer (Guzmán, 2003; Velasco et al., 2012).

Cannabinoids exert most of their effects by binding to and activating CB_1 and CB_2, two cannabinoid-specific GPCRs (Pertwee et al., 2010). However, different evidences suggest the existence of other non-CB_1/CB_2 cannabinoid receptors, at least in neurons, vascular and immune cells (Kreitzer and Stella, 2009). The orphan receptor GPR55 has been recently proposed to be engaged and activated by certain cannabinoids (Ross, 2009), and some authors propose that it can be considered the third cannabinoid receptor (Baker et al., 2006). However, the controversial pharmacology reported so far for this receptor does not unequivocally support this notion (Ross, 2009; Sharir and Abood, 2010). Independently of whether GPR55 does or does not belong to the cannabinoid receptor family, its involvement in cancer physio-pathology has been clearly demonstrated. Thus, GPR55 promotes cancer cell proliferation (Andradas et al., 2011; Piñeiro et al., 2011), migration and invasion *in vitro* (Ford et al., 2010) (chapter 3 of this Thesis). Moreover, GPR55 induces tumor growth and promotion *in vivo* (Andradas et al., 2011; Pérez-Gómez et al., 2012) (chapter 3 of this Thesis). Considering that GPR55 has an important role in cancer and that this receptor is engaged by some cannabinoids, we aimed at investigating its potential involvement in the effect of THC on the proliferation of cancer cells.

It has been described that cannabinoids exert dose-dependent biphasic effects. For example, they can be both anorexigenic or orexigenic, anxiolytic or anxiogenic, and analgesic or algesic depending on the administrated dose (Sulcova et al., 1998). Similar biphasic effects have been described on the proliferation of certain cancer cells. Thus, while "high" concentrations of cannabinoids exert the well known anti-proliferative actions, "low" concentrations have the opposite effect, increasing cancer cell proliferation rates (Caffarel et al., 2010) (unpublished data from our laboratory). Here, we show that GPR55 mediates the stimulatory effect of THC on cell proliferation via GPR55/CB_1 functional interaction.

Materials and Methods

Cell cultures and viability assays

T98G human glioblastoma and EVSA-T human breast adenocarcinoma cell lines were supplied by ATCC-LGC and HEK293 cells were kindly provided by Dr. Maria Waldhoer (Medical University of Graz, Austria). T98G and HEK293 cell lines were maintained in DMEM supplemented with 10% FBS and 1U/mL penicillin/streptomycin. EVSA-T cell line was maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. All cells were incubated at 37ºC and 5% CO_2.

Before cannabinoid challenge, cells were transferred to a low (0.5%) FBS-containing media overnight and then treated with THC (THC, The Health Concept) or the corresponding vehicle (DMSO) for 36 h. Cell viability was determined by the MTT (3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide thiazol blue) test (Sigma, St. Louis, MO) according to manufacturer's instructions.

Plasmids and transfections

Cells were transfected with a 3xHA-human recombinant GPR55 plasmid (Henstridge et al., 2009), with a $G\alpha_{16}$ vector (kindly provided by Dr. Maria Waldhoer) or with the corresponding empty vector (pcDNA3). Lipofectamine 2000 (Invitrogen, Barcelona, Spain) was used as transfection reagent according to manufacturer's instructions. 24

h after transfection, cells were seeded at a density of 5000 cells/cm^2 for viability assays or prepared for calcium assays.

Genetic knock-down of GPR55 by small interfering RNA (siRNA)

Cells were transfected with specific siRNA duplexes using DharmaFECT 3 as transfection reagent according to manufacturer's instructions (Dharmacon-Thermo Scientific, Lafayette, CO). siRNA for human GPR55 was a SMARTpool from Dharmacon-Thermo Scientific. The sequences were 5'-GAAUUCCGCAUGAACAUCAUU-3', 5'-GAGAAACAGCUUUAUCGUAUU-3', 5'-AAGAACAGGUGGCCCGAUUUU-3' and 5'-GCUACUACUUUGUCAUCAAUU-3'. The non-targeted control sequence was 5'-UUCUCCGAACGUGUCACGUtt-3', from Applied Biosystems-Ambion (Austin, TX).

Real-time quantitative PCR

RNA from cell cultures was isolated with Trizol Reagent (Sigma, St. Louis, MO) including a DNase digestion step, with the recombinant DNase I (Roche, Basel, Switzerland). cDNA was subsequently obtained with Transcriptor Reverse Transcriptase (Roche). Real-time quantitative PCR was performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library (Roche). The primers for human GPR55 were: sense, 5′- CATGTGTTTCTCCAACGTCAA-3 and anti-sense, 5′-TGCGGAATTCTTTGATGACA-3′. Amplifications were run in a 7900 HT-Fast Real Time PCR System (Applied Biosystems, Carlsbard, CA). 18S RNA was used as reference (multispecies primers: sense, 5′-GCTCTAGAATTACCACAGTTATCCAA-3′, antisense, 5′-AAATCAGTTATGGTTCCTTTGGTC-3′).

Calcium assays

Intracellular calcium release was measured by monitoring the fluorescent intensity of a calcium sensitive dye after calcium binding (FLIPR Calcium 4 Assay Kit, Molecular Devices, Sunnyvale, CA). 24 h after cells transfection (see above), cells were seeded in 96-well black bottom-clear plates, coated with 1% poly-D-lysine (Sigma) in 10% FBS-containing media at a density of 40000 cells/well. Before the calcium assay, cells were starved overnight. Cells were then incubated in dye buffer for 1 h at 37ºC. Ca^{2+} mobilization was measured immediately after agonist application (L-α-lysophosphatidylinositol (LPI) (Sigma), THC, 2-arachidonoylglycerol (2-AG) (Tocris Bioscience, Ellisville, MO), CP-55940 (Tocris Bioscience) and DMSO) and recorded for 2 min in a FlexStationII System (Molecular Devices). Maximum minus minimum peak height was used to calculate calcium response.

Statistical analysis

Statistical analysis ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test was routinely used. Unless otherwise stated, data are expressed as mean±s.e.m.

Results and discussion

GPR55 modulates the THC-induce pro-proliferative peak

To analyze whether GPR55 participates in the effect of THC on cancer cell proliferation we selectively knocked-down GPR55 in human T98 glioblastoma cells by small interference RNA (siRNA). First, we observed that THC induced a

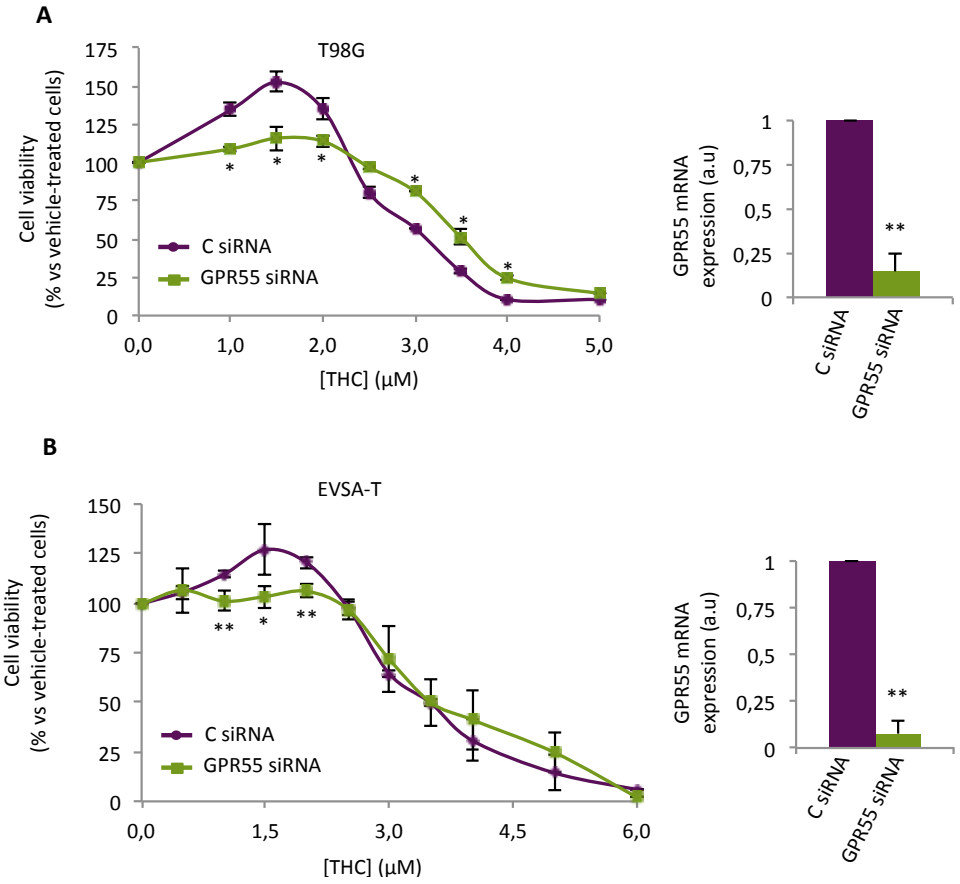

Figure 1. GPR55 knock-down prevents the pro-proliferative response induced by low concentrations of THC. *Cell viability of human glioblastoma T98 (A) and human breast adenocarcinoma (B) cells in response to increasing concentrations of THC. Red lines correspond to cells knocked-down for GPR55 expression (GPR55 siRNA) and blue lines to cells transfected with a control siRNA (C siRNA). Right panels represent the relative GPR55 mRNA levels, as determined by real-time quantitative PCR, after GPR55 silencing. *p<0.05; **p<0.01 vs C siRNA cells.*

biphasic response on cancer cell viability. Thus, low-concentrations (up to 2 µM) enhanced proliferation of T98 cells, while high concentrations (above 2 µM) produced a significant decrease in cell viability. Interestingly, GPR55 down-regulation blocked the proliferative peak induced by low concentrations of THC, and did not modify the anti-proliferative effect produced by higher concentrations (figure 1A). To determine if this was a glioblastoma-specific response, we performed similar experiments in the human breast adenocarcinoma cell line EVSA-T. As in T98 cells, THC induced a bell-shaped response in breast cancer cell viability (stimulation at low concentrations and inhibition at high concentrations) and the proliferative effect was prevented by selective GPR55 knock-down (figure 1B). To further confirm the involvement of GPR55 in THC-induced stimulation of proliferation, we overexpressed the receptor and challenged the cells with different concentrations of THC. GPR55 overexpression resulted in an increased pro-proliferative response to low concentrations of THC, both in glioblastoma (figure 2A) and breast cancer cells (figure 2B). Together, these data strongly suggest that GPR55 mediates the proliferation inducing effect of THC.

THC does not mobilized intracellular calcium via GPR55

We next aimed at determining if THC action was produced by direct activation of GPR55. Whether THC is able to activate GPR55 is still a controversial issue (Sharir and Abood, 2010).

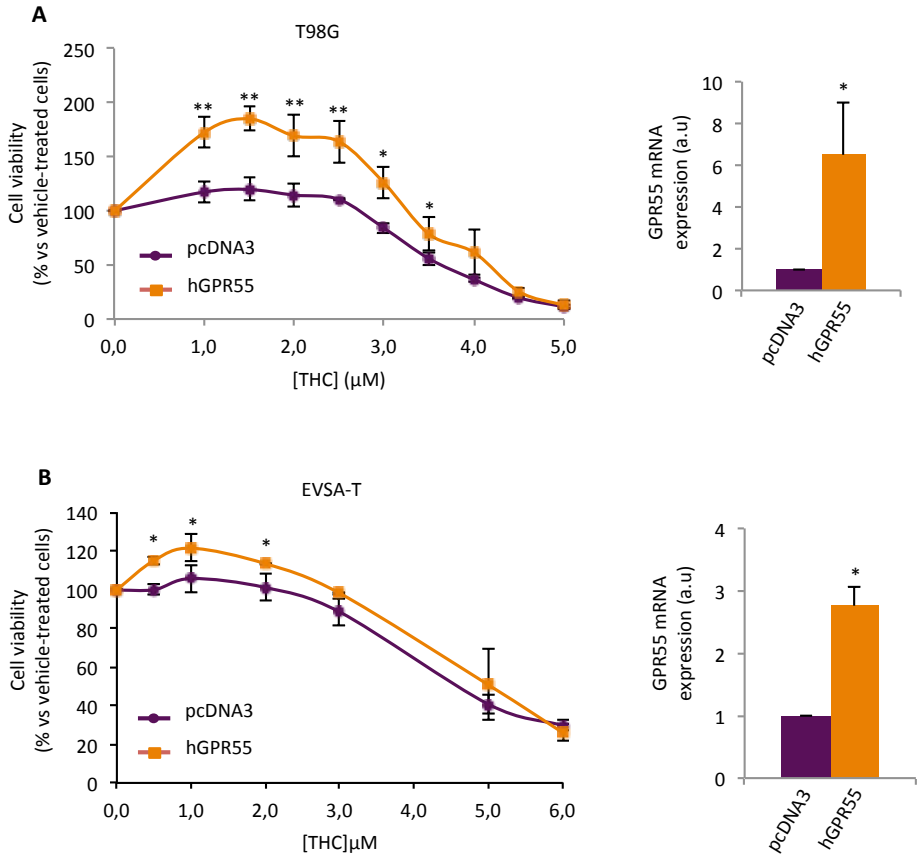

Figure 2. GPR55 overexpression enhances the pro-proliferative response induced by low concentrations of THC. *Cell viability of human glioblastoma T98 (A) and human breast adenocarcinoma (B) cells in response to increasing concentrations of THC. Orange lines correspond to cells that overexpress GPR55 (hGPR55) and blue lines to cells transfected with the control plasmid pcDNA3. Right panels represent the relative GPR55 mRNA levels, as determined by real-time quantitative PCR, after GPR55 overexpression. *p<0.05; **p<0.01 vs pcDNA3 cells.*

Some authors have reported agonistic properties of THC on GPR55 (Lauckner et al., 2008; Ryberg et al., 2007; Yin et al., 2009) while others reported no action of the cannabinoid on this receptor (Kapur et al., 2009; Oka et al., 2010; 2007). To find out what the situation was in our system we used intracellular calcium release as a readout of receptor activation. Since T98 and EVSA-T cells express other receptors that can be activated by THC (CB_1 and CB_2), we performed these experiments in HEK293 cells (which do not express these receptors). First, we confirmed that ectopic overexpression of GPR55 in these cells induced a proliferative effect in response to THC (figure 3A). The proposed GPR55 endogenous ligand LPI induced a significant increase in intracellular Ca^{2+} levels in HEK-GPR55 cells (figure 3B). On the contrary, neither THC or other CB_1/CB_2 mixed agonists (CP-55940 and 2-AG) produced any detectable alteration on intracellular Ca^{2+} (figure 3B). These compounds, however, were able to enhance intracellular Ca^{2+} levels in HEK cells stably overexpressing one of their well-established targets, the CB_1 receptor, which, as expected, did not respond to LPI (figure 3C).

GPR55-CB1 functional interaction modulates proliferation in response to THC

Since GPR55 clearly mediates THC proliferative action, and the best established targets of this cannabinoid are CB_1 and CB_2 receptors, we next aimed at elucidating if there is a functional cross-talk between GPR55 and classical cannabinoid

receptors. It has been previously described that receptor interaction modulates the response and function of the individual receptors (Vischer et al., 2011). In particular, it has been shown that CB_1 can form heteromers with other receptors like the D_2-dopamine receptor or the μ-opioid receptor (Pertwee et al., 2010). In addition, a recent report suggests that CB_1 and CB_2, can form heteromers (Callen et al., 2012). Moreover, an interaction between GPR55 and CB_1 (Kargl et al., 2012) and CB_2 (Balenga et al., 2011) has been described. In all these cases, the association between receptors modulated each receptor's activity. Callen and coworkers reported the presence of CB_1/CB_2 heteromers in the brain that, when activated simultaneously, blocked the activation of the Akt pathway and the neurite outgrowth induced by activation of each receptor independently (Callen et al., 2012). A functional interaction between GPR55 and CB_2 has been described in neutrophils (Balenga et al., 2011). GPR55 activation enhances neutrophil migratory response towards the CB_2 agonist 2-AG, and inhibits neutrophil degranulation and ROS production induced by CB_2 (Balenga et al., 2011).

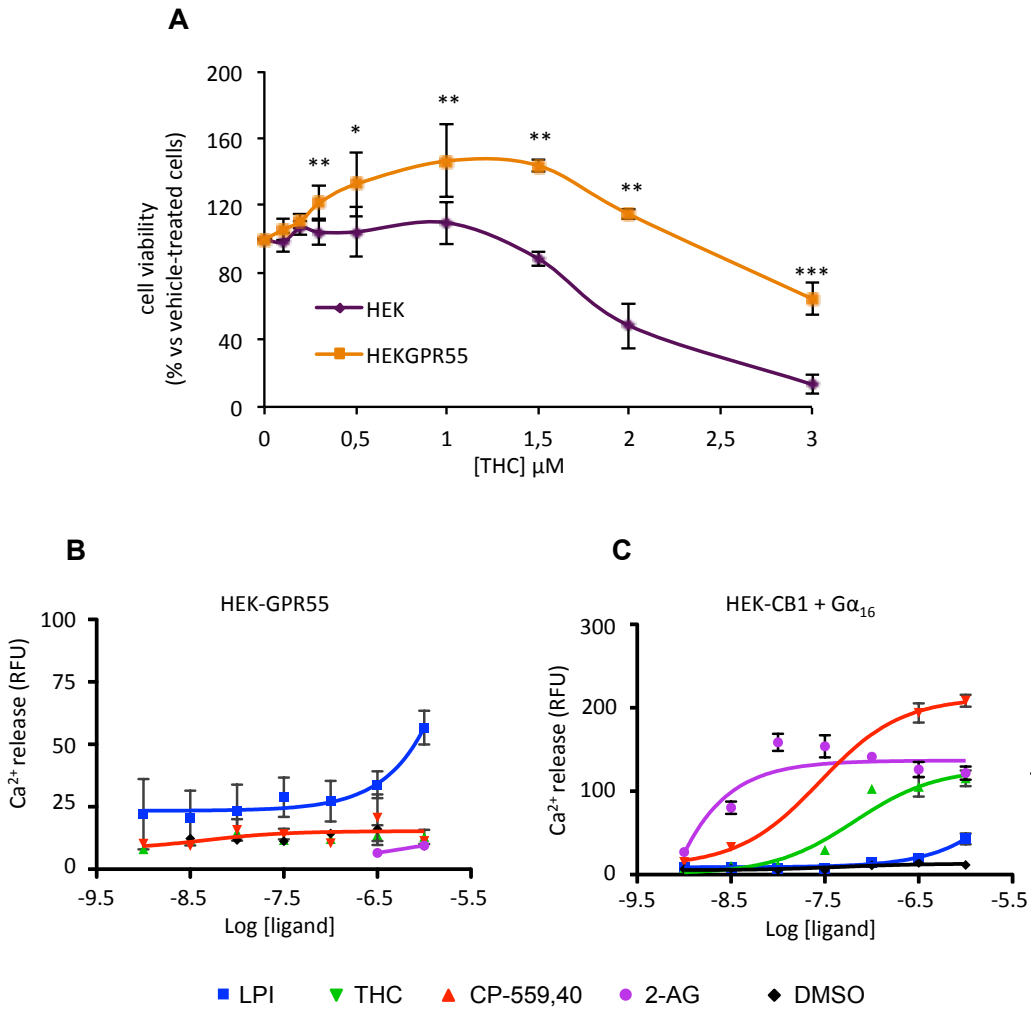

Figure 3. THC does not produce calcium release via GPR55. *A, Viability of HEK293 cells stably overexpressing GPR55 (HEKGPR55) or the corresponding empty vector (HEK) after 36 h of incubation with the indicated concentration of THC. *p<0.05;**p<0.01 vs HEK cells. B, Intracellular calcium levels detected in HEK-GPR55 (B) and HEK-CB_1 (C) cells after treatment with increasing concentration of LPI, THC, CP-55940, 2-AG or the corresponding vehicle (DMSO). Results are expressed as relative fluorescence units (RFU). For experiments with HEK-CB_1 cells, since CB_1 is mainly coupled to Gai proteins, which do not mobilize intracellular calcium, cells were previously transfected with Ga_{16}, a promiscuous member of the $Ga_{q/11}$ family that can bind to most GPCRs, inducing the activation of PLCb and subsequent Ca^{2+} release (Kostenis et al., 2005).*

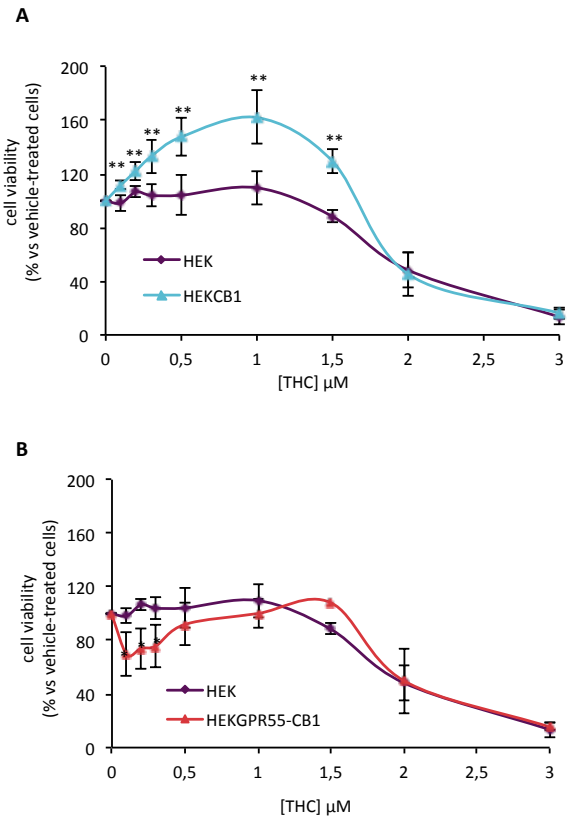

Figure 4. GPR55 and CB1 present a negative cross talk on THC-induced cell proliferation. *Viability of HEK293 cells stably overexpressing CB1 (HEKCB1) (A) CB1 and GPR55 (HEKGPR55-CB1) (B) or the corresponding empty vector (HEK) (A and B) in response to 36 h challenge with the indicated concentrations of THC. **p<0.01 vs HEK293 cells.*

Kargl and coworkers showed that the presence of CB_1 inhibits activation of NFAT, SRE and ERK via GPR55. On the other hand, the presence of GPR55 enhanced the activation of ERK and NFAT mediated by CB_1 (Kargl et al., 2012).

To analyze whether GPR55 functionally interacts with cannabinoid receptors in our context and, if so, if this interaction is relevant in the response to THC, we performed cell viability experiments in HEK293 cells stably expressing GPR55, CB_1 or GPR55 and CB_1. As described before, overexpression of GPR55 enhanced the proliferation induced by THC (figure 3A). The same effect was observed when CB_1 was overexpressed (figure 4A). Interestingly, simultaneous overexpression of both receptors abolished the proliferative peak induced by THC (figure 4B). These data indicate that CB_1 and GPR55 functionally interact to control cell proliferation upon THC treatment, and suggest that CB_1/GPR55 interaction status may be the molecular explanation underlying the biphasic effect of THC on cancer cell proliferation. Further experiments will be performed to confirm this hypothesis and to determine, for example, what are the specific signaling pathways activated by GPR55, CB_1 and GPR55/CB_1 in the pro- and anti-proliferative action of THC; if the functional interaction is the consequence of a physical contact between receptors or to a downstream signaling crosstalk; what is the contribution of other well-established targets of THC (mainly CB_2), etc.

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GENERAL DISCUSSION

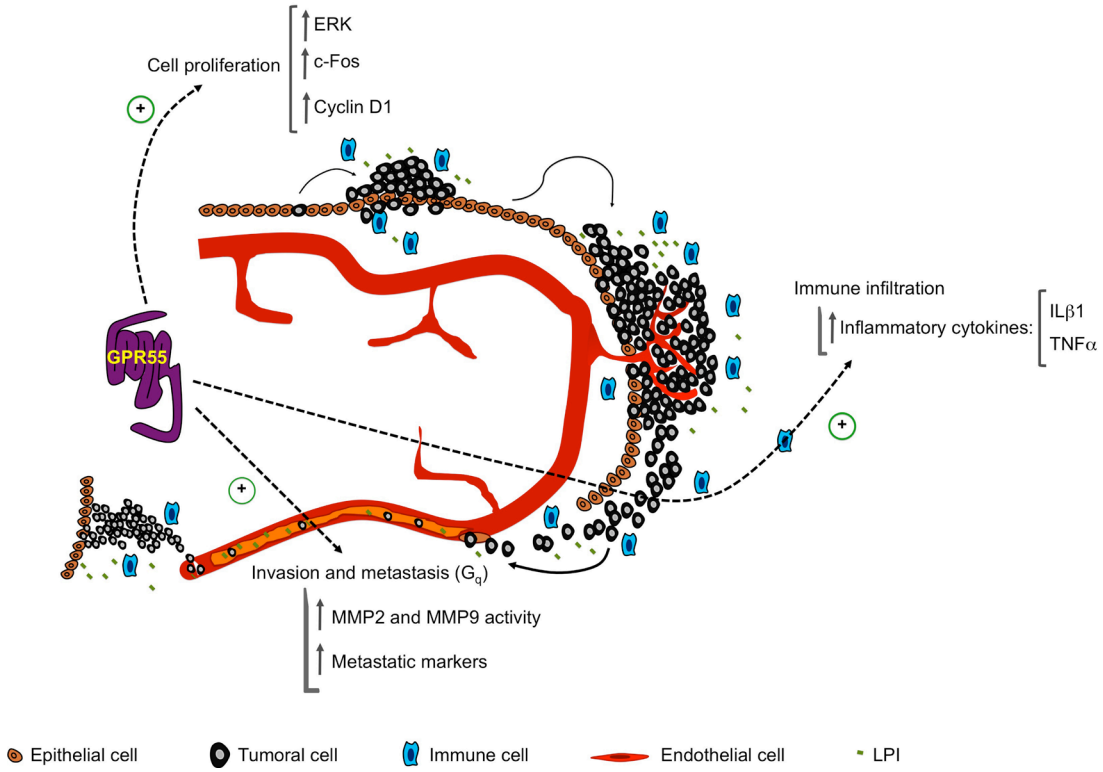
G protein-coupled receptors (GPCRs) represent the largest superfamily of cellular receptors. They control a whole range of physiological functions, and their deregulation contributes to many human diseases. Although they represent together with enzymes the most common pharmacological target of currently prescribed drugs, to date only a small percentage of GPCRs are being therapeutically exploited in cancer. Well characterized GPCRs as well as orphan receptors have been linked to cancer initiation and progression (Dorsam and Gutkind, 2007; Lappano and Maggiolini, 2011). Elucidating their specific mechanism of action and identifying additional GPCRs involved in cancer development is therefore a reasonable strategy to fight against this pathology. Results from this Thesis reveal that the orphan GPCR GPR55 plays an important role in cancer physio-patology. Specifically, we demonstrate that GPR55 promotes tumor generation and progression by conferring proliferation, migration and invasion advantages on cancer cells (Discussion figure 1). Together, these results point to GPR55 as a new potential target in oncology as well as a potential biomarker associated to poor prognosis.

GPR55 is expressed in tumor cells

First of all, by analyzing human cell lines and tumor biopsies from patients, we show that GPR55 is expressed in a wide variety of human cancers. Moreover, the expression of the receptor was significantly increased in tumoral tissues as compared with healthy tissues, and in highly aggressive vs poorly aggressive tumors. These data indicate that GPR55 expression may be used as a potential biomarker in cancer with prognostic value. Aberrant GPCR expression associated to tumor progression has been previously described in human cancers, which promotes tumor progression (Dorsam and Gutkind, 2007; Li et al., 2005; Wu et al., 2012). For example, the receptors for the lysophospholipids LPA and S1P are overexpressed in tumors from different origins such as breast, gastric, thyroid or ovarian cancers (Murph et al., 2006; Murph and Mills, 2007). In addition, cannabinoid receptors, also activated by lipidic compounds, are upregulated in many tumors such as lymphomas, hepatocellular carcinomas, pancreatic adenocarcinomas and breast cancers among others (Caffarel et al., 2012; Malfitano et al., 2011). These evidences point to the existence of a lipid-evoked control of cancer physiopathology. According to this idea, LPI/GPR55 could be a component of this oncogenic lipid circuitry in cancer progression. To confirm the relevance of GPR55 as a prognostic biomarker, the expression of the receptor should be characterized in a greater number of human tumor samples. We are currently studying the expression of GPR55 in a large battery of human breast cancer biopsies from primary tumors as well as from the metastatic lesions. Complete clinical information about these samples (hormone and HER-2 receptor status, histological grade, patient disease-free and overall survival, etc.) is available, and therefore we expect to be able to establish associations between GPR55 expression and patient prognosis in the next future.

GPR55 promotes cancer cell proliferation

We found that GPR55 confers a proliferative advantage on cancer cells from different origins, including gliomas, breast adenocarcinoma and squamous skin cell carcinoma, both *in vitro* and *in vivo*. Additional results obtained by Piñeiro and coworkers and by our group confirmed these observations *in vitro* in prostate, ovarian (Piñeiro et



Discussion figure 1. Role of GPR55 in cancer development*. GPR55 is expressed by tumor cells and promotes cancer cell proliferation by activation of the ERK/MEPK cascade, cancer cell invasion via G_q, migration and metastasis in vivo by activating MMP2 and MMP9 and by increasing the levels of other metastatic markers. In addition, GPR55 promotes tumor immune infiltration by upregulating pro-inflammatory cytokines.*

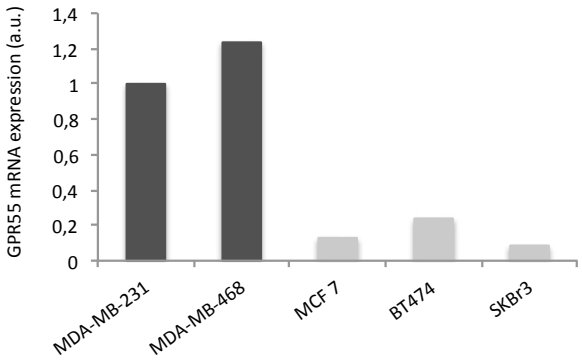
al., 2011) and pancreatic cancer cells (our groups unpublished results), which suggests that the enhanced proliferation induced by GPR55 is general rather than cell-type specific.

It is important to point out that the effects on cell proliferation described here relied upon modulation of the levels of GPR55, without application of any ligand. The enhancement in cancer cell proliferation could be explained either by ligand-independent constitutive activation of the receptor or by the production of GPR55 ligands by tumor cells. Although constitutive activation of GPR55 in cancer cells cannot be ruled out, our data and the data obtained by other groups suggest that cancer cells are exposed to an endogenous tone of the putative GPR55 ligand LPI. Thus, it has been shown that fibroblasts and epithelial cells generate mitogenic LPI upon Ras induced transformation (Falasca and Corda, 1994; Falasca et al., 1998). In addition, either pharmacological (this Thesis) or genetic (Piñeiro et al., 2011) blockade of the main enzyme responsible for LPI synthesis (cytosolic phospholipase A2 (cPLA2)) inhibited the effects of GPR55 on cell proliferation. Moreover, high levels of LPI have been detected in ascites and plasma from patients with ovarian cancer compared with healthy controls (Sutphen et al., 2004; Xiao et al., 2001). Interestingly, the most potent LPI species in terms of activation of GPR55, arachidonoyl-LPI (Oka et al., 2009), is the most increased LPI species in these patients (Sutphen et al., 2004). Together, these results suggest that the release of LPI from cancer cells may be a strategy to stimulate cancer cell proliferation. Similarly, the lysophospholipids LPA (Sutphen et al., 2004; Xiao et al., 2001) and S1P (Hong et al., 1999) are frequently elevated in cancer patients.

These lysophospholipids are potent mitogenic factors that, by activating their cognate receptors (LPA and S1P receptors), stimulate cancer cell proliferation, among other functions, thereby promoting tumor progression (Dorsam and Gutkind, 2007; Lin et al., 2010; Pyne and Pyne, 2010). Once again, these data collectively suggest that LPI/GPR55 are part of a complex lipidic system that controls cell proliferation and is altered in cancer.

Results obtained from the chemically-induced skin carcinogenesis model show that GPR55 expression is predominantly associated to the basal layer of the epidermis, which is a highly proliferating poorly-differentiated compartment of the skin. Preliminary data from our group suggest a similar expression pattern in breast cancer. Normal breast is formed by lobules and ducts, a structure made of two cell layers: one in contact with the light of the lobules and ducts, made of luminal epithelial cells, and one made of myoepithelial cells, juxtaposed to the basal membrane (Rakha et al., 2008). Recent molecular studies have established a strong association between aggressiveness and the basal-like genotype (Bertucci et al., 2012; Rakha et al., 2008) (see below). Interestingly, we have observed higher GPR55 expression in basal-like (MDA-MB-231 and MDA-MB-468) than in luminal-like breast cancer cell lines (Ford et al., 2010) (Discussion figure 2). Moreover, preliminary data from our group show that, as in skin cancer, GPR55 expression is mostly associated to this basal, highly proliferative and poorly differentiated compartment.

It has been described that GPR55 activates the ERK/MAPK cascade in different cell systems, including HEK293 cells (Anavi-Goffer et al., 2011; Henstridge et al., 2010; Kargl et al., 2012; Oka et al., 2009; 2007), osteoclasts (Whyte et al., 2009), endothelial cells (Zhang et al., 2010), U2OS cells (Kapur et al., 2009; Kotsikorou et al., 2011) and the pheocromocytoma cell line PC12 (Obara et al., 2011). The ERK/MAKP cascade is one of the main regulators of cell proliferation (McCubrey et al., 2007). Results described in this Thesis show a link between GPR55 and ERK on cell proliferation. Thus, GPR55 expression is associated with active ERK and the subsequent increase of the transcription factor c-Fos, and cyclin D1 *in vitro* and *in vivo*. Furthermore, Piñeiro and co-workers have also reported that GPR55 controls ERK activity (Piñeiro et al., 2011). The authors observed a clear ERK activation after LPI stimulation in prostate and ovarian cancer cells, an effect that was blocked after down-regulation or pharmacological inhibition of GPR55 (Piñeiro et al., 2011). Although these results support that GPR55 controls cell proliferation through the ERK/MAPK cascade, the involvement of other players cannot be ruled out. For example, the pro-tumorigenic PI3K/Akt signaling pathway, which is over-activated in many cancers (Vivanco and Sawyers, 2002), has been implicated in GPR55 action on cancer cell proliferation. Thus, Piñeiro and co-workers observed that activation of GPR55 induces phosphorylation



Discussion figure 2. GPR55 expression is increased in basal-like breast cancer cell lines. *Analysis of GPR55 expression by real-time quantitative PCR in five breast cancer cell lines: two basal-like cancer cell lines MDA-MB-231 and MDA-MB-468, and three luminal cell lines MCF-7, BT474, SKBr3.*

of Akt and that GPR55 knock-down decreased the basal levels of activated Akt (Piñeiro et al., 2011).

GPR55 drives breast cancer cell invasion via G_q

The potential of cancer cells to disseminate throughout the body and generate metastases is one of the main determinants of cancer aggressiveness and patients' overall survival. As described in the Introduction, GPR55 couples to $G_{12/13}$ and G_q proteins (Henstridge et al., 2011; Ross, 2009), heterotrimeric proteins that are involved in cancer invasion and metastasis (Dorsam and Gutkind, 2007). It has also been described that activation of GPR55 stimulates small Rho GTPases (Henstridge et al., 2011), proteins that modulate cytoskeleton organization, motility and cell adhesion, among other functions (Karlsson et al., 2009). These observations suggest a potential role of GPR55 in invasion and metastasis. Results presented in this Thesis confirm that GPR55 promotes cancer cell invasion and migration *in vitro* and *in vivo*. This idea was strengthen by Falasca's and Ross' groups. Thus, GPR55 down-regulation in prostate cancer cells reduced the number of colonies formed in soft agar, and the colonies that grew were less organized than those formed by GPR55-expressing cells (Piñeiro et al., 2011). Additionally, overexpression of GPR55 in the poorly metastatic breast cancer cell line MCF-7 induced migration in cell culture, and activation with LPI enhanced the migration of the GPR55-overexpressing cells towards serum (Ford et al., 2010).

The mechanisms underlying the pro-invasive potential of GPR55 are still poorly understood. In this Thesis, we demonstrate that the invasion induced by GPR55 in MDA-MB-231 breast cancer cells is mediated by G_q proteins. Our results suggest that activation of MMPs is also involved in the GPR55 effect on cancer cell invasion and/or migration. MMPs promote tumor angiogenesis, invasion and metastasis by inducing the degradation of the extracellular matrix, the disruption of cell-cell interactions, the release of angiogenic factors and the processing of membrane-associated growth factors and cytokines (Gialeli et al., 2010; Hua et al., 2011). In fact, tumors tissues, plasma, serum and urine from patients with different cancers, such as breast cancer, pancreatic cancer, lung cancer, colorectal cancer, bladder cancer and ovarian cancer, present elevated levels of MMPs (Roy et al., 2009). We found that the activity of MMP2 and MMP9, two MMPs often enhanced in cancer, was significantly reduced in GPR55 knocked-down cells. Interestingly, it has been reported that the lysophospholipids LPA and S1P increase cancer cell invasion (Murph et al., 2006; Panupinthu et al., 2010) by, at least partially, activating MMPs (Kim et al., 2011; Komachi et al., 2012). Further experiments should be performed to widen our knowledge on the molecular mechanisms underlying the pro-invasive and pro-migratory actions of GPR55.

Most primary tumors display organ selectivity for the generation of metastases. As described in the Introduction, GPCRs can participate in this selectivity. The metastatic organ releases specific GPCR ligands, which serve as chemoattractant signals for cancer cells that express those GPCRs located in the primary tumors or in the circulation. Our results demonstrate that LPI acts as a chemoattractant, inducing the migration and invasion of GPR55-expressing MDA-MB-231 breast cancer cells in culture. It would be interesting to evaluate whether this effect is produced *in vivo* as

well. We also found that GPR55 enhances the levels of the metastatic markers ANGPTL4, CXCL1 and MMP1. Growing evidences support the idea that tumor cells acquire genetic alterations that can be encompassed in a gene-expression profile or signature, that in turn can predict the risk of metastatic recurrence and favor organ specific metastases (Nguyen and Massagué, 2007). For example, some genes such us epiregulin, CXCL1, MMP1, MMP2, the cell adhesion molecule SPARC, VCAM1 receptor and ANGPTL4 have been implicated in breast cancer metastasis to the lungs (Minn et al., 2005; Padua et al., 2008). The modulation of a specific set of metastasis-related genes by GPR55 suggests that GPR55 could favor a gene profile that confers cancer cells a higher and probably organ-specific metastatic potential.

We and others (Ford et al., 2010) have found that GPR55 is abundantly expressed in the highly metastatic breast cancer cell line MDA-MB-231 compared with the poorly metastatic breast cancer cells MCF-7, BT474 and SKBr3. Moreover, we found that GPR55 levels are higher in basal-like breast cancer cell lines than in luminal cells (Discussion figure 2). According to their molecular portraits, breast cancer can be classified into five groups: estrogen receptor positive (ER+), that is divided into luminal A and luminal B; HER-2 positive, characterized by the overexpression of Erbb2; basal-like, and normal breast-like tumors (Perou et al., 2000). Basal-like breast cancers, among other features, are characterized by an increased expression of genes associated with motility and invasion and, consequently, patients with this type of tumors present shorter overall metastasis-free survival (Bertucci et al., 2012). Our data suggest a connection between GPR55 expression and the basal genotype that is especially important due to the lack of efficient therapies for these patients. If confirmed in a relevant number of cell lines and human samples, GPR55 would turn into a new target for the management of these highly aggressive tumors.

Involvement of GPR55 in other hallmarks of cancer: sustained angiogenesis and altered immune surveillance

Although there is no solid evidence demonstrating the involvement of GPR55 in other hallmarks of cancer, it is tempting to speculate that this receptor may play a role in tumor angiogenesis and anti-tumor immune surveillance. As described in the Introduction, GPR55 is expressed in endothelial cells (Henstridge et al., 2011). Activation of GPR55 in human microvasculature endothelial cells (HMVEC) stimulates endothelial proliferation and migration, upregulates VEGF and induces tube formation *in vitro*, all of them important events during angiogenesis (Zhang et al., 2010). These results indicate that GPR55 participates in the control of the angiogenic process and suggest that it may also modulate tumor-related angiogenesis. In our glioblastoma xenograft studies we observed no differences in vascularization of tumors from cells expressing high or low levels of GPR55, but these results do not allow ruling out a role of GPR55 in tumor angiogenesis. For example, in our experimental model, GPR55 knock-down was specifically directed to human tumor cells and did not affect the expression of the receptor in mouse endothelial cells, which are the main responsible for the neovascularization of tumors. Additionally, we found that GPR55 modulates the levels of MMP1. It has been described that this metalloproteinase can activate PAR1 receptors on endothelial cells, inducing their proliferation, migration and neoangiogenesis (Goerge et

al., 2006; Pei, 2005).

Increasing evidence points to a crucial role of the tumor microenvironment, mainly composed by stromal and immune cells, on tumor progression and aggressiveness. GPR55 is expressed in immune cells and tissues (Henstridge et al., 2011) and different studies have reported an important role of the receptor in immune responses. Thus, GPR55 modulates the response to inflammatory and neuropathic pain by controlling the levels of various cytokines (Staton et al., 2008). It also promotes chemotaxis and regulates ROS production in neutrophils (Balenga et al., 2011). It might also have anti-inflammatory effects. Thus, inflammatory agents induce the release of NGF in mast cells, which is blocked by GPR55 activation (Cantarella et al., 2011). It is well described that TPA topical application in the mouse skin induces, besides hyperproliferation, local inflammation (Abel et al., 2009). Results presented in this Thesis show that GPR55-deficient mice present a reduced number of inflammatory infiltrating cells in the dermis. Moreover, the increased levels of inflammatory cytokines produced by TPA topical application were not observed in GPR55 KO mice (Discussion figure 1). Together, these data suggest that the pro-oncogenic properties of GPR55 may include the alteration of the tumor-associated inflammatory response.

GPR55 participates in the effect of THC on cancer cell proliferation

Although the best characterized ligand of GPR55 is LPI, it has been reported that some cannabinoids are able to engage and activate this receptor (Baker et al., 2006; Sharir and Abood, 2010). It is also well described that cannabinoids from different origins (synthetic, endogenously produce and plant-derived) exert anti-tumoral effects *in vitro* and *in vivo* (Hermanson and Marnett, 2011; Velasco et al., 2012). Most of these effects are produced by activation of CB_1 and CB_2 cannabinoid receptors, but the involvement of additional targets cannot be ruled. Thus, we analyzed the potential participation of GPR55 in the effect of THC on cancer cell proliferation. Specifically, GPR55 seems to mediate the proliferative effect induced by low concentrations of THC. Interestingly, this participation is not restricted to a specific type of tumor cell and it was demonstrated in breast cancer cells, glioblastoma cells (this Thesis) and pancreatic adenocarcinoma cells (our group's unpublished data). Whether THC is able to bind and activate GPR55 is still a controversial issue (Sharir and Abood, 2010). Our data suggest that THC does not release intracellular Ca^{2+} via GPR55. Preliminary results point to the existence of different GPR55/CB_1 interaction status that may lead to the activation of different signaling pathways and, therefore, to the different biological responses (enhancement vs inhibition of cell proliferation) produced by different concentrations of the same compound (THC). The relevance of GPCR heteromers in regulating cell signaling has been previously reported. In the context of cannabinoid receptors, several authors have confirmed that CB_1/dopamine receptors, CB_1/opioid receptors (Pertwee et al., 2010), CB_1/CB_2 (Callen et al., 2012), CB_1/GPR55 (Kargl et al., 2012) and CB_2/GPR55 (Balenga et al., 2011) heteromers signal through different signaling pathways than each individual receptor. Experiments are currently in progress to confirm the functional interaction of CB_1 and GPR55 in cancer cells and to determine if that interaction is produced by physical contact of the receptors or by a downstream signaling crosstalk. The participation of CB_2 in this complex scenario is also being tested.

An alternative possible mechanism of GPR55 activation by THC involves heterotrimeric G protein-independent pathways. The most studied alternative signaling pathway is mediated by β-arrestin (Maurice et al., 2011). Two reports have used the recruitment of β-arrestin to determine receptor activation by THC, though the results are controversial. One of them did not find β-arrestin recruitment after THC treatment of HEK293 cells overexpressing GPR55 (Kapur et al., 2009), while the other showed that THC behaved as a weak agonist in this assay (Yin et al., 2009). Further experiments should be carried out to determine whether THC activates GPR55 and induces β-arrestin signaling. This has been however demonstrated in closely related GPCRs. For example, CB_1 mediates downstream signaling through β-arrestin, thereby activating Src, MEK1/2 and ERK (Ahn et al., 2013); LPA induces NFκB activation and IL-6 expression through β-arrestin (Sun and Lin, 2008); and CB_2 upregulates the serotonin receptor 5-HT_{2A} by a mechanisms that requires β-arrestin (Franklin et al., 2012). It would be interesting to analyze, for example, whether GPR55 (via β-arrestin) modifies the expression levels of classical cannabinoid receptors.

Although our results on the involvement of GPR55 in the biphasic effect of THC on cancer cell proliferation are still very preliminary, they represent the first approach to unmask the molecular bases underlying cannabinoid bell-shaped actions in cancer cells, which have been extensively documented in other various biological settings. For example, biphasic cannabinoid effects have been reported on fear coping strategies (Metna-Laurent et al., 2012), visual accuracy (McLaughlin et al., 2005), anxiety (Rey et al., 2012) or feeding behaviors (Sulcova et al., 1998). In these processes, at least part of the dual action of cannabinoids relies on the selective binding of different doses of the drug to activatory/glutamatergic or inhibitory/gabaergic neuronal terminal (Bellocchio et al., 2010; Rey et al., 2012). This provides another line of support to the exciting notion that different pools of a cetain GPCR located on different cell subpopulations can signal in a different manner, thus further increasing the complexity of these fascinating signaling platforms.

CONCLUSIONS

Results obtained in this Thesis allow us to conclude that:

1. The orphan receptor GPR55 confers pro-oncogenic advantages on tumor cells *in vitro* and in mouse cancer models by promoting their proliferation, migration and invasion. Consequently, elevated expression of GPR55 in human tumors is associated to high aggressiveness and poor prognosis.

2. GPR55 participates in the proliferative effect of THC on cancer cells as produced by low concentrations of the cannabinoid.

Together, these conclusions point to GPR55 as a new biomarker with possible prognostic value, and as a new therapeutic target in oncology.

CONCLUSIONES

Los resultados obtenidos en esta Tesis nos permiten concluir que:

1. El receptor huérfano GPR55 proporciona ventajas pro-oncogénicas a las células tumorales, induciendo su proliferación, migración e invasión tanto *in vitro* como *in vivo*. En consecuencia, niveles elevados de GPR55 en tumores humanos se correlacionan con una mayor agresividad tumoral y un peor pronóstico de los pacientes.

2. GPR55 participa en el efecto del THC sobre la proliferación de células tumorales, concretamente en el aumento de la proliferación inducido por bajas concentraciones del cannabinoide.

En conjunto, nuestros datos sugieren que GPR55 podría ser una nueva herramienta con valor pronóstico y una nueva diana terapéutica en cáncer.

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